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Sarah Kristine Slanger
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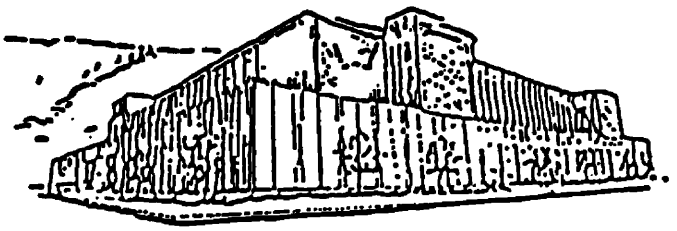

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DETECTION OF MULTIPLE DRUGS IN HUMAN HAIR FROM SEVEN DRUG CLASSES USING ONE DIGESTION/EXTRACTION PROTOCOL

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1999

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Abstract

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KP

Analysis of the hair fiber for scheduled drugs, therapeutic drugs, or other xenobites which bind to the hair shaft has become a useful tool in forensic investigations. Specific digestion-extraction procedures have been developed for a variety of basic and acidic drugs, however, development of a comprehensive, general screening method to confirm the presence of multiple drugs in different drugs classes using one analytical technique has not been reported. This paper explores the development of a digestion-extraction procedure for drugs in the following classes: amphetamine-like compounds, benzodiazepines, opiates, cocaine and cocaine analogs, barbiturates, tricyclic antidepressants, and the drug propoxyphene. The development of a sensitive analytical procedure to determine and confirm drug presence in the hair fiber and a novel approach to differentiate between endogenous and exogenous drug binding are discussed.

Throughout a two year period 26 postmortem human hair samples were collected from county coroners across the state of Montana. Routine toxicological analysis of urine and blood samples were performed prior to hair analysis; however, the results of this screening were not known until the conclusion of the project. Hair analysis revealed the presence of 42 drug confirmations, 10 of which were not identified in the urine, and 25 which were not identified in the blood. These data strengthen the validity of the developed digestion-extraction and analytical procedures and support the role of hair analysis as a supplemental screen in forensic toxicology.

A novel approach to differentiate between endogenous and exogenous drug exposure was explored using anti-opiate monoclonal antibodies. The study was initially designed using a rat model; however to expand the findings in the preliminary study, a similar protocol was applied to human hair. Positive and negative controls, in addition to two case samples containing hydrocodone, were sectioned at 5 microns, mounted on microscope slides, and immunocytochemistry was performed. The human hair controls differentiated between the staining patterns of drug versus non-drug controls, determined by the characteristic brown staining of diaminobenzidine. While preliminary investigations reveal this protocol as a viable method of exogenous drug detection, further research is needed to explore the possibilities of using antibodies as a method of detection for exogenous drug contamination in additional drug classes.

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ACKNOWLEDGMENTS

Throughout the course of this research project I have had the distinguished pleasure of working with many talented professionals in the scientific community. I would like to extend my deepest thanks to the following individuals for helping make my work possible:

Jim Hutchison: for introducing me to the wonderful science of Forensic Toxicology and being the backbone of this project;

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Thank you!

Chapter 1. Background

I. *Introduction*

Human hair serves a variety of functions for the human body ranging from environmental protection to thermal insulation. On a macro-scale, hair provides a visual differentiation between humans, based upon color, shape, and length of hair. On a micro-scale level, hair is a complex network of fibers and vacuoles that have the capacity to store information about an individual's lifestyle. This lifestyle information is derived from xenobites bound in the hair fiber. Analysis of the hair fiber for scheduled drugs, therapeutic drugs or other xenobites which bind to the hair shaft, provides an extended exposure history which other biological samples do not provide. Pubic, beard, auxiliary, and head hair have the capability to bind drugs within the hair fiber. Head hair is the most common hair type studied in research facilities across the United States. (1,2)

The simplest model proposed for the incorporation of drugs into hair theorizes that drugs enter the fibril by passive diffusion from the bloodstream into the growing hair cells at the base of the hair follicle, and then become tightly bound in the interior of the hair shaft during keratogenesis. However, more complex models which consider drug deposition from sweat, distribution volume, pharmacokinetics and drug lipophilicity have been invoked to explain variables such as the concentration differences in hair of various individuals receiving the same dose of drugs, as well as to clarify why drug concentration and metabolite ratios in hair are quite different from those found in blood. (3)

In 1990 the Society of Forensic Toxicologists (SOFT) issued a consensus opinion

summarizing the current applicability of hair analysis for testing for drugs of abuse:

> ".....the use of hair analysis for workplace drug testing [is] premature and interpretation of test results [are] insupportable with current information. Hair is a useful specimen in forensic investigations when supported by other evidence of drug use."

At the time, no generally accepted procedures for hair analysis were available and the accuracy, precision, sensitivity, specificity, and cutoff levels defining positive and negative test results were not established. Reference materials (to standardize analytical methods) were not available and many important questions remained regarding incorporation, retention, stability and analytical detection of drugs in hair. This prompted an explosion of research over the next several years.

Specific digestion-extraction procedures have now been developed for a variety of basic and acidic drugs including phencyclidine, methadone, codeine, flunitrazepam, heroin, cocaine, cannabis, ketamine, carbamazepine, nicotine, and morphine. (4,5,6,7,8,9,10) These procedures have become more reliable, allowing researchers to address more complex issues such as dose-response and binding kinetics, but development of a comprehensive general screening method to test for several drug classes using a single digestion-extraction technique has not been reported. A cost-effective, time-efficient screening method for hair would be valuable to the forensic community, both as a supplemental toxicological screen and as an investigative tool to future researchers.

The development of a general screening procedure for drug analysis in hair is the principle purpose of this report. The research involves the following specific aims:

1. Development of a digestion procedure for:

a). fortified hair; negative (drug free) hair soaked in a drug solution

b.) non-fortified hair, or case samples;

2. Development of an extraction procedure for the following drugs and drug classes: amphetamine/amphetamine analogs, benzodiazepines, opiates, cocaine, propoxyphene, barbiturates, and tricyclic antidepressants;

3. Development of a sensitive analytical procedure for the presumptive determination and the confirmation of drugs in human hair.

II. *History of Hair Analysis*

Since the early 1960's, scientists have used hair as a biological tool of discovery. The morphology and physiology of hair provides a unique matrix for recording exposure information of environmental substances over a longer time period than other biological samples (such as blood or urine). The importance of hair analysis increased when researchers realized that hair could store heavy metals along the hair shaft in a time-course dependant manner with most recent exposure being recorded in the proximal hair segment. The time-course relationship of metal deposition along the hair fiber sparked interest in the possibility of obtaining the drug history of a chronic drug user in the hair shaft. Most abused drugs and several therapeutic drugs have been detected in the hair of chronic users. (11) Hence, hair analysis has been proposed to investigate drug abuse for epidemiological, clinical, administrative and forensic purposes.

In the 1980's, William Baumgartner and associates began investigating which drugs bind in/on hair. As more information became available regarding how drugs associate with the hair fiber, scientists began to question decontamination processes in

relation to exogenous drug exposure. Investigators began to focus more attention on wash protocols and the binding kinetics for specific drugs or drug classes in an attempt to validate the usefulness of hair as a biological tool in forensic cases.

The toxicological specimens analyzed in the most medico-legal cases are biological fluids such as blood, urine, and vitreous humor. However, in some instances, hair is the preferred matrix for forensic investigation. Historic hair, such as that of Napoleon, has been analyzed to determine if the cause of his death was acute arsenic poisoning. (12) In this case, hair was the only biological sample available for analysis (versus blood, urine, or tissue). In other instances, hair analysis is preferred because it is less invasive than urine or blood analysis, as in cases where children are involved. (13) Hair analysis is beginning to play a greater role in criminal investigations as scientists learn more about the drug-hair interactions that make identification of drug exposure possible. To elucidate the interactions of drugs and hair, investigators are further exploring the relationships between and within the micro-structure of the hair fibril. The applicability of hair analysis to a wide range of drugs is beginning to come into focus as more scientists experiment with digestion-extraction models which target a particular structural component of hair, such as melanin.

III. *Characteristics of Hair*

A. Anatomy

Human hair is a fiber-like substance which covers a majority of the human body. It serves to protect, insulate, and stimulate responses on the skin. Hair is composed of cylindrical structures or shafts made up of tightly compacted cells which grow from small

sac-like organs called follicles. The diameter of individual hair shafts in man range from 15 to 120 micrometers (*u*m), Figure 1.

Each hair shaft is made up of three distinct types of cells. The cuticle, the outer most layer of the hair shaft, is scale-like in nature. The single layer of elongated, overlapping individual cells is 0.5 to 1.0 *u*m thick and approximately 45 *u*m long. The mechanical stability provided by the cuticle anchors the hair shaft in the follicle and protects the inner regions of the hair shaft by acting like plated armor. The cuticle also hinders exposure of the hair shaft to exogenous substances which could degrade the integrity of the inner hair. The cuticle may become damaged or destroyed by chemicals, heat, light, or mechanical injury, resulting in a less intact (frayed) structure toward the distal end of the shaft, Figure 2. (14)

The cortex lies under the cuticle. The cortex forms the bulk of the hair shaft and is composed of keratinized cells which form fibers approximately 100 *u*m long. Cortical cells are spindle-shaped cells which contain fibrous proteins that enclose small spaces called fusi. In the living portion of the hair root, these sacs are filled with fluid. Air replaces the fluid as the hair shaft matures and dries out. Pigment granules are also found in cortical cells. Melanin is the primary pigment of hair, skin, and eyes. Melanin is synthesized in specialized organelles called malanosomes which are located within melanocytes in the hair bulb. The enzyme tyrosinase converts the amino acid tyrosine to melanin, Figure 3.

On the most interior portion of the hair shaft is the medulla. Medullar cells are initially loosely packed. As the hair grows, these cells dehydrate and shrivel up to leave a

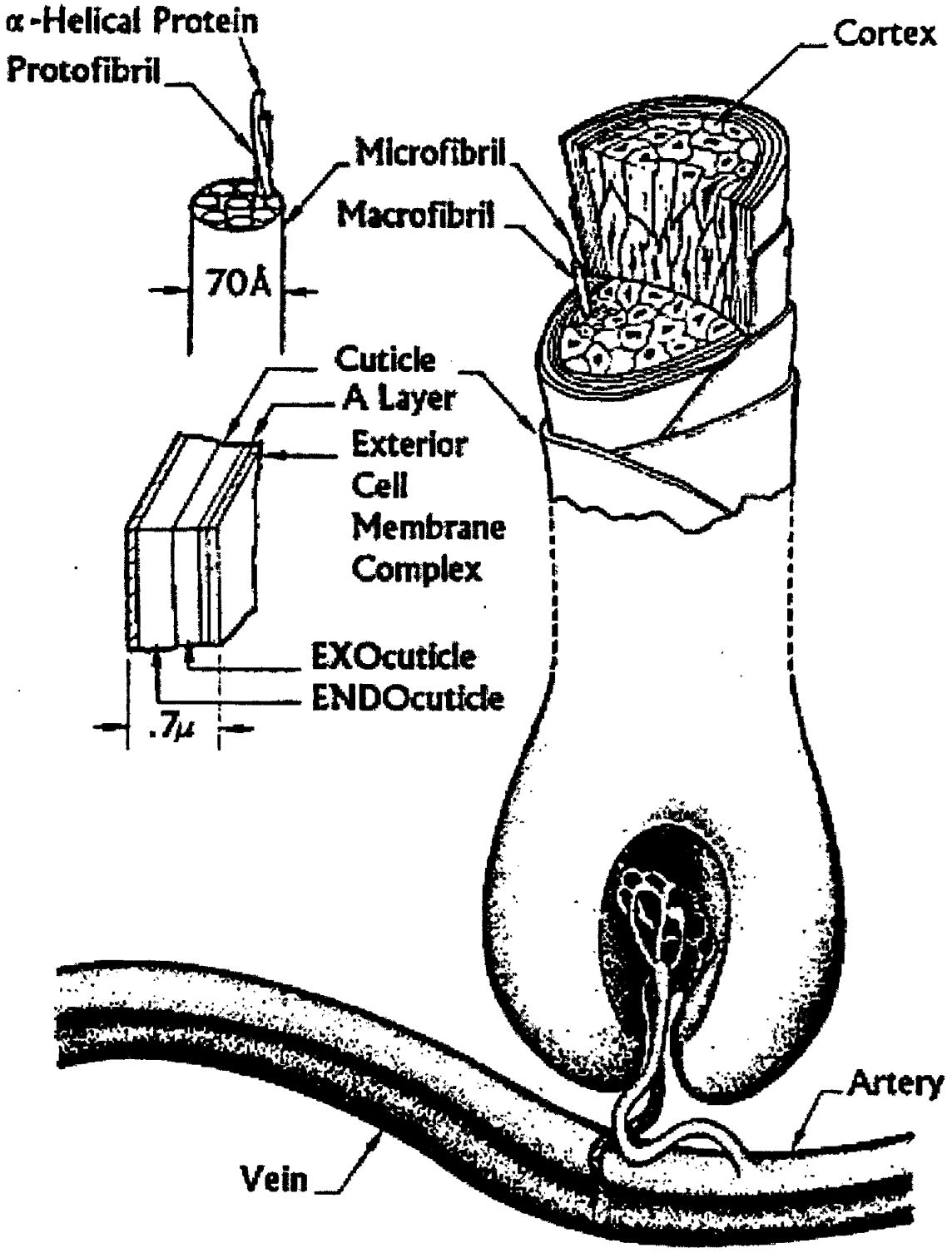


Figure 1. Anatomy of a human head hair follicle. *Handbook of Analytical Therapeutic Drug Monitoring and Toxicology.* CRC Press Incorporated. 1997

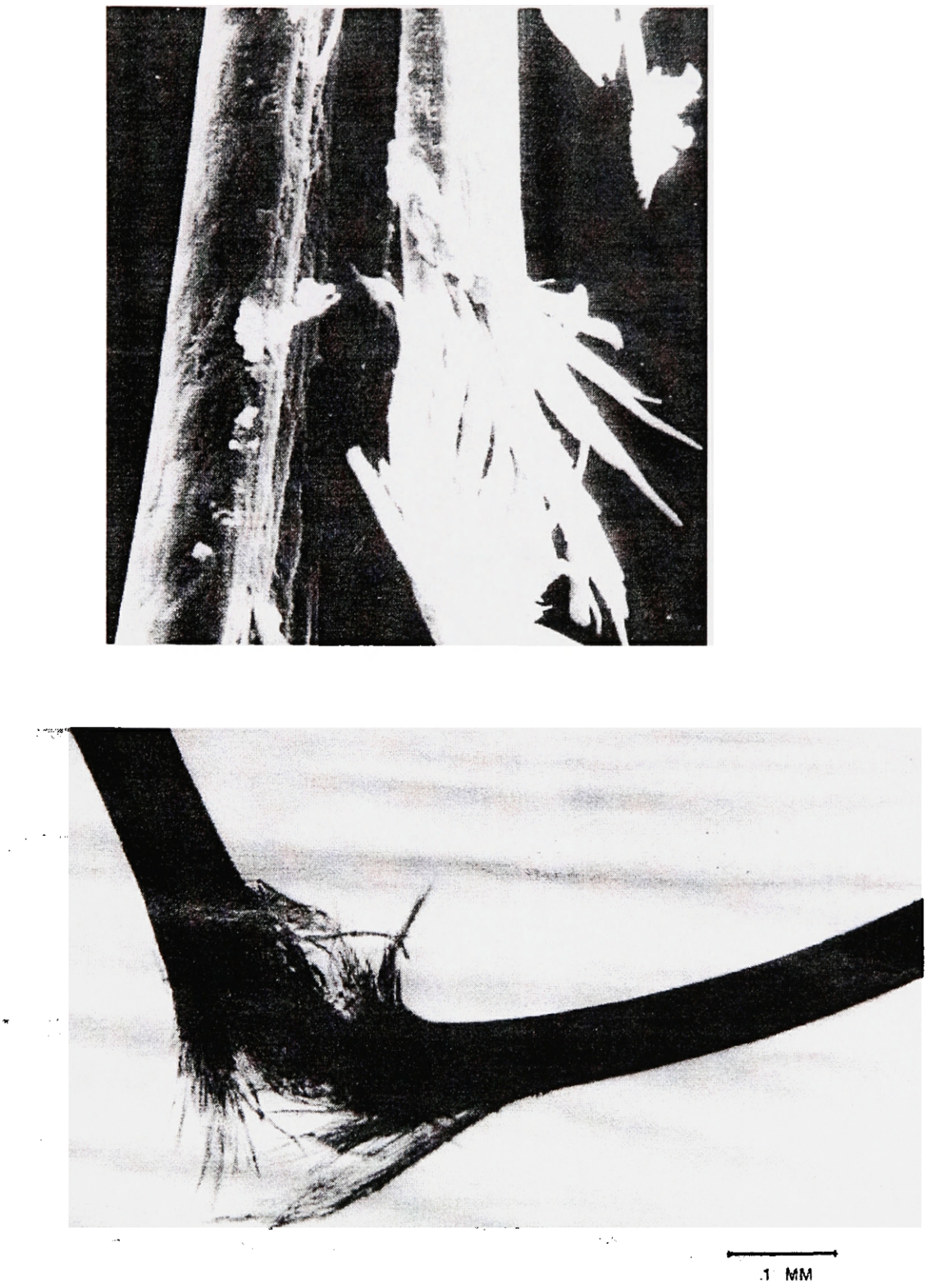


Figure 2. Scanning electron micrograph of a split hair. Note cortical cell fragments, which appear as "broomlike" fractures. *Chemical and Physical Behavior of Human Hair.* 3rd Ed. Springer 1994.

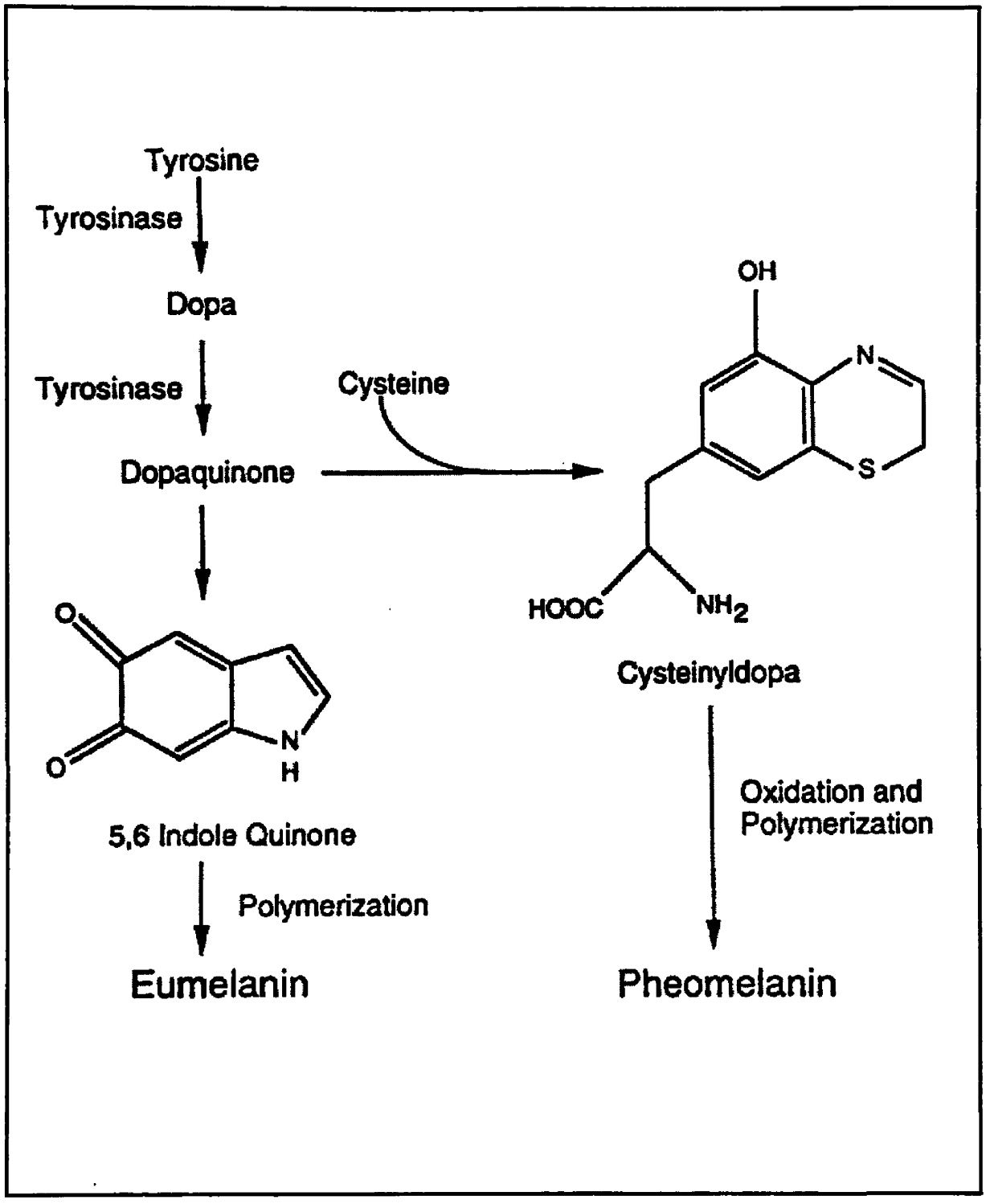


Figure 3: Proposed biosynthetic pathways for eumelanin and pheomelanin. *Drug Testing in Hair (1996)*

series of vacuoles along the fiber axis. The medulla functions to provide the major pathway of diffusion into the fibers of the cortical region.

Mature hair fibers contain at least two of the three structures described above. In human hair, medullar cells comprise only a small percentage of the mass of the hair shaft and may be completely absent, continuous or discontinuous along the center of the hair, or double (as in human beard hair).

The micro-structure of hair is essentially a cross-linked, partially crystalline oriented polymeric network. Human hair contains relatively large amounts of the following amino acids: hydrogen side-chain (glycine), hydroxyl side-chain (threonine), primary amide (aspartic and glutamic acid), mono-basic (lysine), thiol (cysteine), and phenolic (tyrosine). Hair also contains proteins (65-95%), lipids (1-9%), and water (15-35%). (14)

The outer surface of the mature hair shaft has three distinguishable zones, Figure 4. Synthesis and orientation of the hair take place in zone one, the zone of differentiation and biological synthesis. Keratinization occurs at the bulb end, zone two. In this region, the hair becomes stabilized through the formation of cysteine cross-links between keratin molecules. Zone three is the permanent hair fiber, which is characterized by the dehydrated nature of the cuticle.

B. Types of Hair

Hair on the human body is classified into three groups of hair fibers based upon the differences in length, texture, color, diameter, and shape. Vellus hair is very fine, short (less than 1 cm), non-pigmented hair that is on the seemingly hairless parts of the body

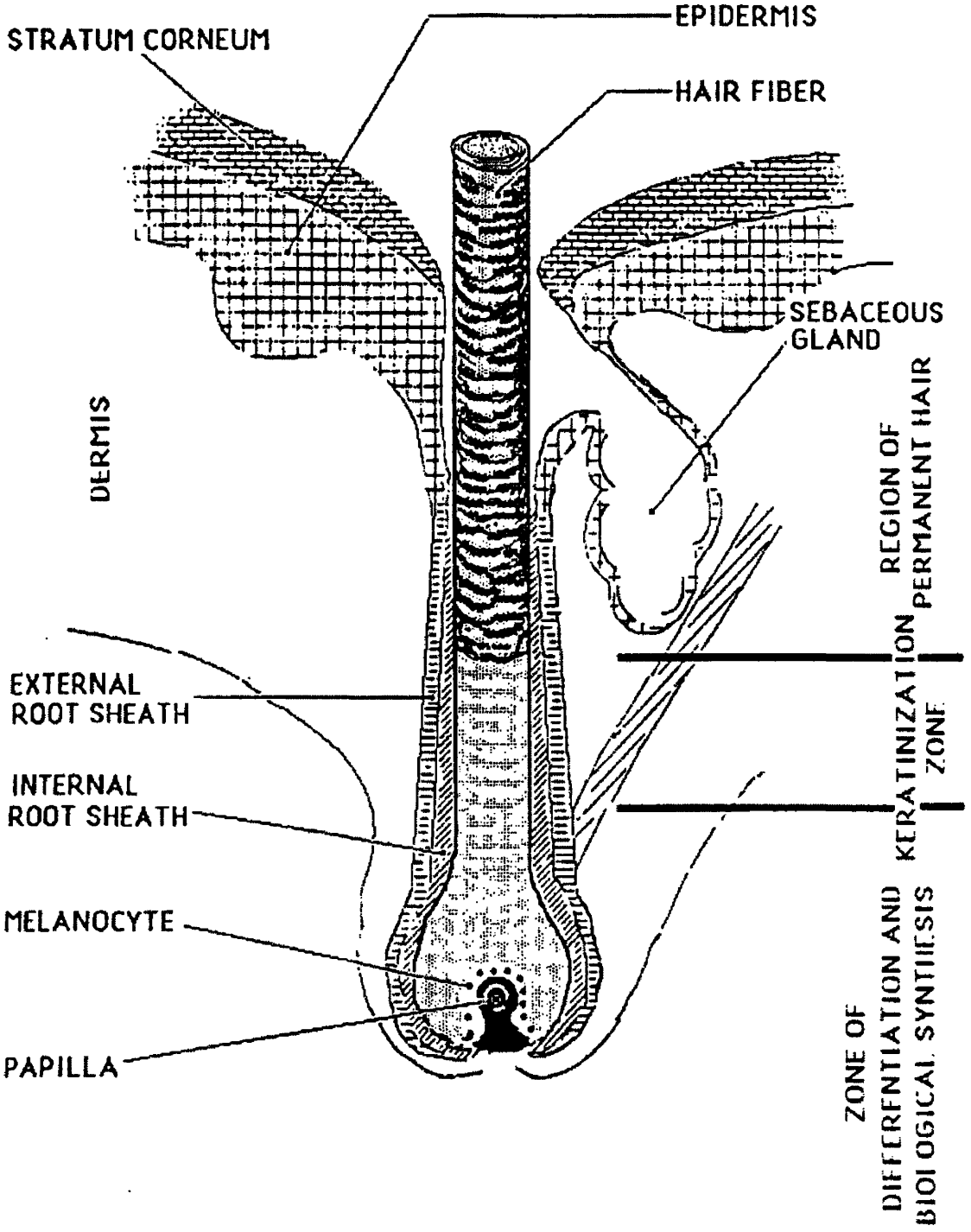


Figure 4. Pilosebaceous unit illustrating a hair follicle with its fiber and the different zones of growth and structural organization as the fiber emerges through the scalp. *Chemical and Physical Behavior of Human Hair.* 3rd Ed. Springer 1994.

such as eyelids, forehead, and the bald scalp. Course hair, called terminal hair, is longer (greater than 1 cm), and contains pigment. Terminal hair is found on the "hairy" parts of the body; the scalp, beard, eyebrows, and eyelash. The third classification of hair, intermediate hair, is intermediate in length (approximately 1 cm) and shaft size, and is found on the arms and legs of adult humans.

These three types of hair are produced from three corresponding follicles. Intermediate hair is produced by non-sexual hair follicles found along the arms and legs in males and females. Non-sexual follicles are not influenced by hormonal changes and do not change hair type after puberty. Ambosexual hair follicles cover the axilla, pubic, and temple areas of the male and female body. These follicles are sensitive to hormonal changes. Vellus hair produced by the ambosexual hair follicle in the armpit and pubic areas of the body changes to terminal hair with the onset of puberty. In the temple region, terminal hair changes to vellus hair to result in the characteristic reshaping of the facial outline. Sexual hair follicles are the third type of follicle and are unique to males. These follicles are found in the beard, ear, nose, chest, and abdomen areas of the male. At puberty, these follicles respond to increased androgen levels by changing production of vellus hair to terminal hair. Interestingly, baldness in males is not a disease, but a condition in which the sexual follicles in the anterior vertex area of the scalp revert from producing terminal hair to vellus hair.

C. Growth

Hair moves through three phases of growth before it reaches maturation. The first phase is the actual growth phase of fiber and is called anagen. Anagen is further

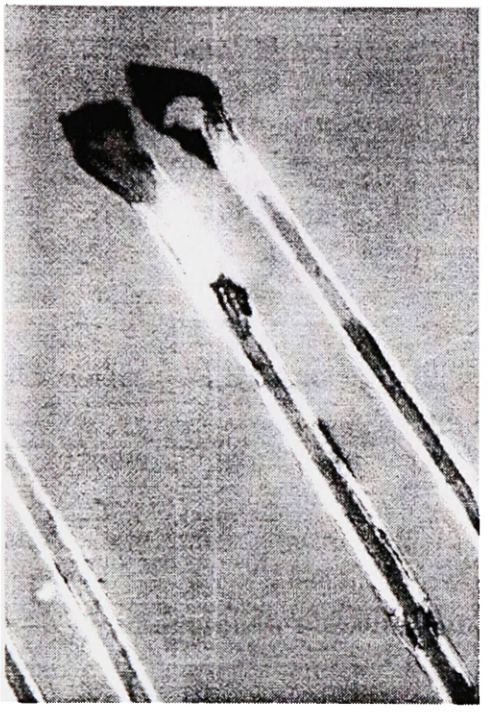
subdivided into six substages (I to VI). Substages I through V are collectively called *proanagen* and are defined by progressively higher levels of new hair tip position within the follicle. The VI substage, *metanagen*, is defined by the emergence of the hair shaft above the skin surface. Human scalp hairs have a relatively long hair cycle with the anagen stage typically ranging from 2 to 5 years. (15) The hair then enters into the second stage of the growth cycle known as catagen.

The catagen stage of the growth cycle is a degenerative stage in which cell division stops and the base of the hair shaft becomes fully keratinized and forms the dry, white node characteristic of a "club" hair, Figure 5. (14) As the bulb degenerates, the follicle becomes considerably shorter. Human scalp hair generally has a short catagen phase of several days.

Telogen represents the final stage of the growth cycle, the resting stage. Normal telogen is approximately 3 months in adult scalp hair. Once a hair follicle has made the transition to telogen, its existing hair will never grow longer. The club-shaped proximal end within the hair follicle will typically be shed from the follicle during telogen or during the subsequent anagen.

Morphological changes in the hair shaft may be tracked throughout the growth cycle, Figure 6. During anagen, the follicle penetrates deepest into the skin, typically to the level of subcutaneous fat. Catagen is heralded by pyknotic changes in the nuclei of hair keratinocytes followed by apoptosis within the transient portion of the follicle. The entire transient portion of the follicle is reabsorbed except for the basement membrane (glassy membrane), which folds up like an accordion bellows, causing follicular retraction

A.



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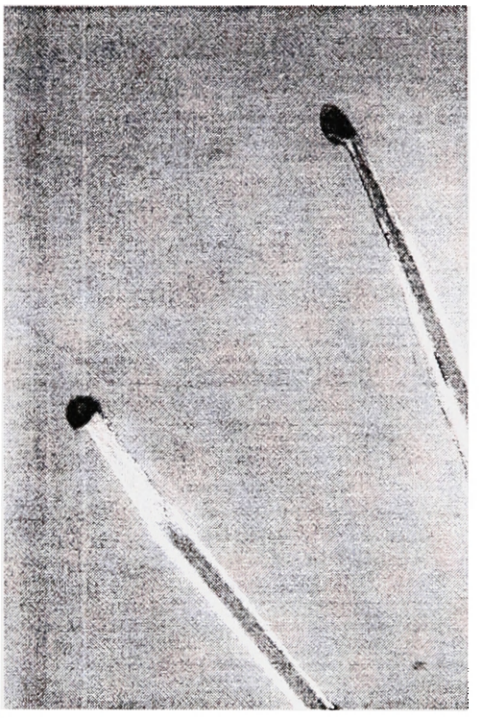


Figure 5. Light micrographs of plucked hair fibers in A. anagen stage and B. telogen stage. *Chemical and Physical Behavior of Human Hair.* 3rd Ed. Springer 1994.

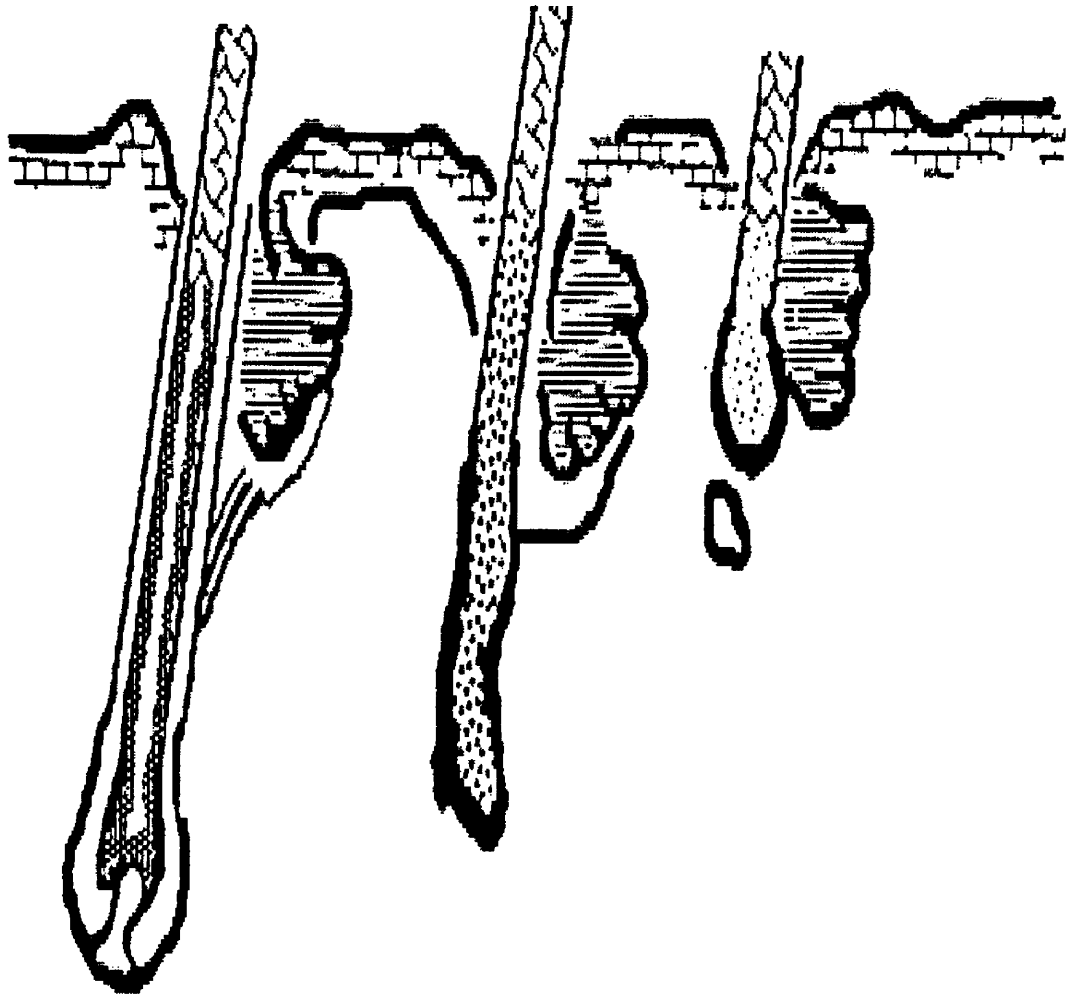


Figure 6. Schematic illustrating the three stages of growth of the human hair fiber. *Chemical and Physical Behavior of Human Hair.* 3rd Ed. Springer 1994.

and associated higher placement of the papilla remnants (located at the base of the root bulb) within the dermis. The inner root sheath, which disintegrates during anagen at the level of the sebaceous duct, is totally absent from the telogen follicle.

A complete description of growth regulation for hair is still unclear. However, several relationships have been recognized. Simple observation leads us to believe that there is a genetic program that specifies normal growth parameters for hairs located on different anatomical locations of the human body such as the eyebrows, scalp, auxiliary, and facial areas. This type of regulation is the innate programming of the follicle and likely specifies such features as hair cycle duration, rate of hair production, type and quality of hair, and sensitivity to other growth hormones. Additionally, hair growth may be greatly influenced by various hormones including thyroid hormones, corticosteroids, and sex hormones; for example, androgens are known to up-regulate the pubic, axillary, and beard hair but down-regulate genetically predisposed scalp hair follicles in common baldness. In addition, morphometric analysis of the hair follicles from the middle back region of spontaneous anagen mice that received parathyroid hormone suggests that this hormone-related peptide may be a major factor responsible for controlling the hair cycle in this species of rat. (16) The growth rate of each type of hair is different both between classes (terminal, axillary, vellus) and within a class. The difference between classes may be explained in part due to the different functions the respective hair type serves. For example, if the function of hair is to warm the body, it can be anticipated that long, thick hair will occur on the head of the body where humans lose 90% of their body heat.

1. Segmented Hair Analysis

While the variance of growth rates for hair type is complex, it is this feature of hair that offers researchers the possibility to obtain a timetable of drug exposure. (17) The principle of a chronological interpretation of drug concentrations in hair segments is based on the following assumptions detailing segmental hair analysis:

- the drug is incorporated from the bloodstream into the hair root within a limited time period
- there is a constant (and known) hair growth rate, as well as a fixed position and stable concentration of drugs in the growing hair shaft
- negligible additional competitive incorporation mechanisms which would lead to additional deposition sites along the hair shaft
- negligible physical or chemical elimination of the drug from the hair shaft (through normal hygiene practices or cosmetic applications) that would lead to a change of the initial segmental concentrations during the hair lifetime

Under the above conditions, the time of the drug consumption can be calculated from the drug position in the hair shaft using the following equation:

$$Ti = (Ts - 1i)(Vh-1r)/(Vh-To)$$

Where Ti=time of the drug intake; Ts= time of the hair sampling; To= time between incorporation of the drug into the hair root and appearance at the skin surface; 1i= distance of the drug position in hair from the proximal end of the hair sample; 1r= length of the residual hair shaft from the skin surface after sampling; and Vh= hair growth rate. (18) This model indicates that the accuracy of hair analysis for the estimation of Ti increases

with decreasing segmental length.

The analysis of segmented hair analysis should be interpreted very cautiously. The variability in growth rates, drug deposition along the hair shaft from sweat/sebum, drug redisposition within/along the hair fiber, variable exposure to weather (photochemical destruction of hair pigments), and undefined growth parameters for postmortem samples prevented segmental analysis in this research study.

D. Hair Pigmentation

Melanin is one of the factors believed to be involved in binding drugs into hair. Melanocytes located above the follicular papilla in the matrix area of the follicle produce the pigments. Pigment-laden extensions of the melanocytes move out along the differentiating hair keratinocytes. Each follicle produces only one of the two types of pigment, eumelanin or pheomelanin. These pigments are polyanionic polymers with a high content of negatively charged carboxyl groups and o-semiquinones. Eumelanin is a brown-black polymer of dihydroxyindole subunits synthesized from the precursor amino acid tyrosine. This melanin has a quinone-like structure and is highly anionic. (4) Pheomelanin is also synthesized from the precursor tyrosine; however, biosynthesis involves the incorporation of a cysteine residue into benzothiazine derivatives. Pheomelanin is a reddish-yellow pigment.

Melanins are incorporated among the keratin fibrils of hair. (6) The varying shades of natural hair color are determined by the quantity of these two pigments in the hair fiber. Substances with cationic groups under physiological conditions, such as amines and metals, bind to eumelanin by ionic interactions, Figure 7. (5) The ionic bonding is

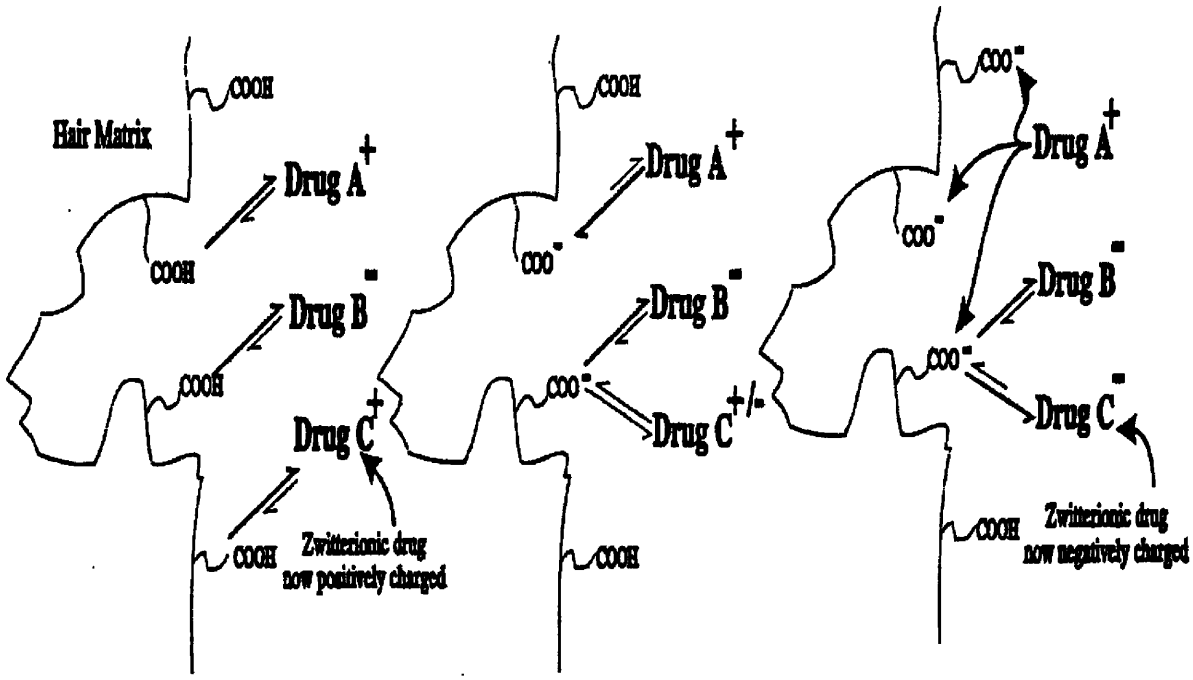


Figure 7. A model of drug incorporation into hair. Drugs A through C could represent a competing cation that could occupy a binding site on the hair and block a drug molecule from binding. The first schematic is pH< 4 (hair predominately neutral, drugs bind via hydrophobic actions, the second schematic is pH 7 (hair negatively charged, some drugs become zwitterionic, drugs bind/repel via ionic interactions), third schematic is pH 9 (hair more negatively charged, some drugs become negatively charged, drugs bind/repel via ionic interactions). *Drug Testing in Hair.* CRC Press Incorporated 1997.

presumably strengthened by non-electrostatic contributions such as van der Waal's forces at the conjunctions of the aromatic rings in the xenobite and the aromatic indole nuclei of the melanin. (6) Green and Wilson have suggested that following penetration of the melanocyte, drugs interact with a melanosomal protein and melanins. (19) This binding is complex. Scatchard analysis shows more than one type of binding for individual substances, and implicate both cooperating binding mechanisms and stearic factors. (6) Drugs which are cations at physiological pH 7.4 are incorporated into hair to a greater extent than those drugs which are anions. (5) This finding supports the role of eumelanin in drug binding. The role of pheomelanin in drug binding has not been established.

E. Effect of Hair Cosmetics on Drug Analysis in Hair

One issue of concern for drug analysis in hair is the change in the drug concentration induced by popular cosmetic treatments of hair such as bleaching or permanent waving. The products used for these treatments are generally strong bases which affect the stability of incorporated drugs and cause alterations of the fibers at an ultra structural level. (20) Partial or complete loss of drug substances may result from a "more permeable" hair fiber. Although "permed" and bleached hair shows an enhanced absorption capacity, investigators revealed that an increased risk of confirming drugs from exogenous origin with these treatments does exist, but the risk is not particularly severe. (21)

Chapter 2. Development of Decontamination, Digestion, Extraction and Analytical Procedures

I. Drugs Included in Study

In 1996 the Montana State Crime Laboratory concluded a two-year study undertaken to determine whether or not MCA 61-8-402 (prohibitive Driving Under the Influence of Drugs legislation) should be amended. In this study, drug levels from 744 individual subjects were recorded, as well as the age, gender, race, and blood alcohol concentration. Drug occurrences by class were as follows: barbiturates (3%), benzodiazepines (15%), amphetamine and amphetamine analogs (11%), opiates (7%), tricyclic or other antidepressants (5%), cocaine or cocaine metabolites (3%). (32) Drugs included in this study protocol were based upon the above statistics detailing the prevalence of abuse for Montana as well as the ease of chromatography/spectrometry of the drug (based upon literature searches) and the assays available for the presumptive screening instrument, the Enzyme Multiplied Immunoassay Technique (EMIT). In addition to the above six drug/drug classes, we also included propoxyphene (which is a synthetic opiate).

A. Length of Detection for Drugs in Hair

The time between drug incorporation and appearance of the drug in the hair fiber (at the skin surface) will vary between drugs based upon unique chemical properties of each drug (such as pH, bioavailability, stearic factors, competitive mechanisms, and elimination rates). However, using an average growth rate of hair and the distance a growing fiber must travel before emerging from the skin (the distance between the matrix cell level and the level of complete keratinization, 1.2-1.5 mm), the To, time between

incorporation of drug and appearance at skin surface, can be estimated between 9 and 14 days. (33) In contrast to physiological expectance, in most cases the drug is first detected between 6 hours and 4 days after application. The short appearance time To (in shaved, not pulled hair) is also confirmed by experiments with guinea pigs, rabbits and rats. (34,35,31)

B. Limit of Detection for Instrumental Analysis

The large number of drugs in each drug class prevents the generalization of an overall limit of detection (LOD). However, when possible, 1 or 2 prototypical drugs from each class were analyzed to become more familiar with parameters such as LOD for the instrument. All limits of detection are derived from fortified hair samples and are in reference to the detection of the drug by the gas chromatography-mass spectrometry (GC/MS). The LOD's are calculated using the following example calculation:

Step 1 100ng drug/ 1 milligram hair sample = 1000 ng drug total (analyzing 10 milligrams hair)

Step 2 1000 ng drug/350 micro-liters methanol (reconstitution) = 2.856 ng drug/ mirco-liter methanol

Step 3 2 micro-liters methanol/ injection = 5.71 ng drug / injection on GC/MS

Several experiments were designed in this manner and their results are summarized in Table 1. Following Table 1 is a graphical representation of the LOD study.

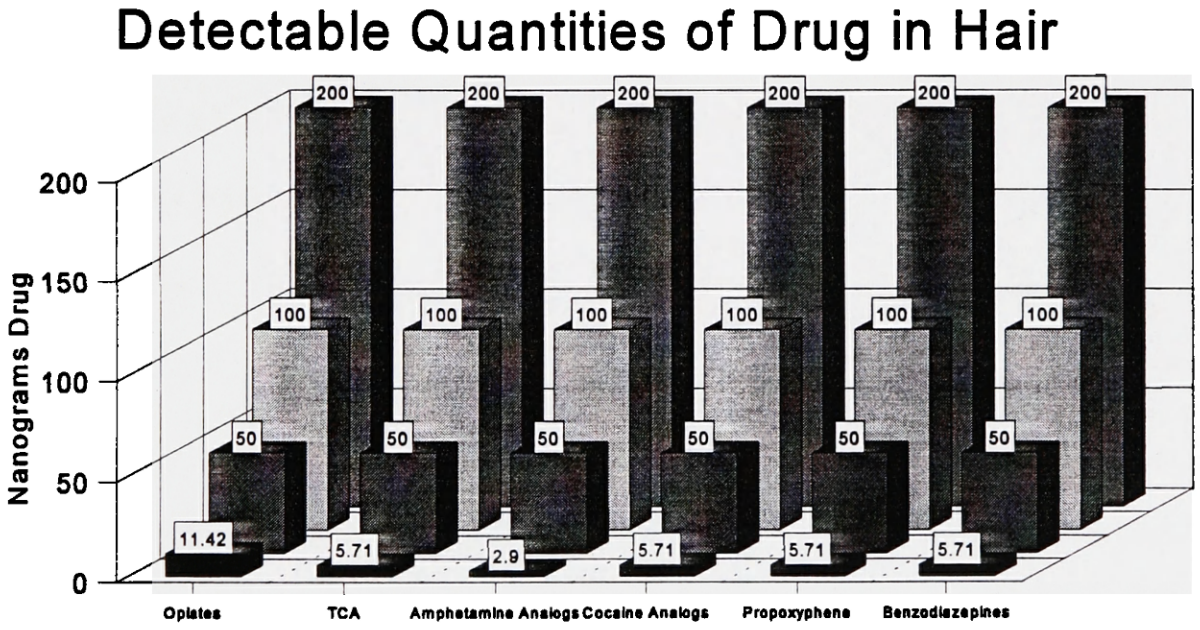
Table 1. Limit of detection for variety of drugs included in the study. Prototypical drugs were chosen from several of the drugs classes of interest to determine a representative limit of detection for analytical purposes.*

DRUG	AMOUNT DRUG DETECTED (NANOGRAMS)
Fentanyl	30
Methadone	30
Oxycodone	30
Codeine	11.42
Benzphetamine (Internal Standard)	11.42
Papaverine	11.42
Amitriptyline	5.71
Imipramine	11.42
Nortriptyline	30
Carbamazepine	30
Fluoxetine	30
Caffeine	30
Propoxyphene	5.71
Tramadol	30
Norpropxyphene	11.42
Amphetamine	11.42
Methamphetamine	5.71
Fenfluramine	2.9
Ephedrine	11.42
Phenteramine	30
Chlorpheniramine	30
Lidocaine	5.71
Flurazepam	30
Midazolam	30
Triazolam	30
Alprazolam	30
Chlordiazepam	30
Chlordiazepoxide	5.71

* LOD's were determined using 10 milligrams of hair fortified with 1000 nanograms of drug.

The information provided by the LOD study serves to initiate a better understanding of the limits of the analytical method. This information must be interpreted with caution, providing only a *representation* of the quantity of a drug which can be detected in human hair. Additional research is needed to further the quantitative description of limits of detection in non-fortified positive human hair samples.

Graph 1: Four series of extractions, beginning at a drug level of 200 nanograms (per 10 milligram hair) and ending at < 15 nanograms, were utilized to estimate the limit of detection for one prototypical drug of 6 drug classes on the GC/MS.



II. Decontamination Procedure

Although exact mechanisms of drug binding in the hair fiber are not known, it is apparent that drug may be deposited in hair by multiple routes including the bloodstream (endogenous), sweat and sebaceous glands, and from environmental exposure (exogenous). (22) The earliest theoretical position explaining the incorporation of drugs

into hair is known as the Entrapment model. In this model, drugs present in the bloodstream are entrapped in inaccessible regions of the hair during the hair growth process. After the hair emerges from the scalp, these "entrapped" drugs form bands along the hair fiber that are in direct proportion to the concentrations present when the hair was formed. This model proposes that the entrapped drugs are protected by the hair matrix from removal or change by the external environment (normal hygiene processes). A second model, the Sweat model, suggests that some drugs may be incorporated into the hair fiber as described above, via the bloodstream. However, water-soluble drugs are also excreted into the sweat and sebum which bathe the hair at the base of the hair shaft. These water-soluble drugs, which are secreted in the sweat and sebum, come in contact with the hair on the exterior of the skin. This model follows several different approaches when compared to the Entrapment model. The Sweat model predicts few or no regions in the hair that are inaccessible to the external environment, and drugs in the hair fiber are deposited from three sources: blood, sweat/sebum, and passive exposure (environmental exposure).

The possibility of exogenous exposure in the forensic setting dictates the need for a decontamination step before the hair sample is digested. Some investigators indicate that exposure from exogenous and endogenous sources can be distinguished through specific washing procedures and the use of mathematical algorithms. (23) However, other investigators report that unless the contamination is very heavy, there is no consensus as to which samples are externally contaminated. Unfortunately, decontamination washes remove endogenous drugs as well as exogenous drug. (24,25) Therefore, we needed to

identify a wash protocol that extracts the least amount of an endogenous drug while still removing drugs on the exterior of the hair fiber.

In this project, five common wash solvents were selected for evaluation based upon their prevalence in published literature: methanol, methylene chloride, MilliQ water, phosphate buffer, and sodium dodecylsulfate. (1) Quantitative data show phosphate buffer and sodium dodecylsulfate extract at least 30% of endogenous drug from the hair fiber in rat models whereas methanol, methylene chloride, and water extract no more than 20% of endogenous drug. Water is the least invasive solvent, extracting approximately 13% endogenous drug (values are expressed as percent decreases in measured concentrations as compared with non-washed hair values in nanogram drug/milligram hair).(26) Based upon these findings, deionized water was selected for the wash protocol in our study (see Appendix One).

III. Digestion

The digestion process refers to the initial stage of hair analysis in which the macro-structure of the hair is degraded by chemical (liquid solvent) or mechanical (pulverized) methods. Chemical solubilization results in a denaturation of cysteine linkages (through reduction) and structural proteins through either high or low pH. The end stage of digestion in this project is determined by the length of contact time between the hair samples and the digestion solvent. In other words, the digestion period is concluded after a specified length of time has passed (3-4 hours). *Complete digestion* refers to the complete breakdown of all hair fibers in the sample of interest as determined by visual observation.

The following digestion techniques were incorporated into the study based upon their prevalence in the literature: acidic (hydrochloric acid), enzymatic (Tris buffer, dithiothreitol, proteinase K, and sodiumdodecyl sulfate), and basic digestion (sodium hydroxide). These three techniques were analyzed to determine which digestion would provide the single best procedure to detect the largest variety of drugs based upon recovery studies of the drugs using spectral data obtained on a GC/MS.

The basic and acidic techniques provided digestion of the hair sample and both these samples were extracted (as described in Appendix 2). The enzymatic technique did not digest the hair sample, even after 24 hours of contact, and therefore was not extracted or further analyzed. A comparison of the spectral data from the basic and acidic samples established the basic digestion procedure as the superior digestion technique by the increased number of drug confirmations. Based upon the results of our initial experiments, we chose to pursue a technique that incorporated a basic digestion of the hair. This finding is supported in the literature when comparing the reproducibility of dissolution and extraction efficiencies of enzymatic and pH related digestions respectively. (27,28)

A. pH Effecting Drug Degradation

To investigate the severity of drug degradation caused by pH extremes, one experiment was designed using drug solutions in place of fortified hair samples. The drug solutions were exposed to 0.01, 0.5, and 1.0 N sodium hydroxide, the basic digestion solvent, for the initial digestion step for 3-4 hours. The drug solutions were then exposed to a pH change, using buffers, to achieve a final pH <6 or a pH>13. This step mimics (in

an extreme example) the possible environment of the hair sample coming from a digestion step with very high pH range, to an extracting environment, with a lower pH range (pH~9). To determined extent of degradation to individual drugs, the results from each analyzed drug solution were compared to non-processed drug solutions prepared at equivalent drug concentrations. Degradation was apparent for cocaine, benzodiapezines, barbiturates, propoxyphene, and amphetamine assays at all pH ranges, as determined by a comparison of EMIT rates (see Chapter 3) of the drug solutions exposed to pH changes versus rates of drug solutions not exposed to any change in pH. However, when the experiment was repeated with the addition of a buffering step (sodium borate solution, pH 9.2) prior to the initial "digestion" of the drug solution, cleaner baselines and sharper spectral peaks were detected on the GC/MS and equivalent rates were observed between "digested" drug solutions and non-processed controls on the EMIT with the higher pH (pH>13) extract versus the lower pH (pH<8) extract.

IV. Extraction

The study investigated solvents that best extracted drugs out of the solublized hair media. These solvents included n-butyl chloride, chloroform, hexane, and chloroform:isopropylalcohol (4:1). In addition to liquid-liquid extraction, solid phase extraction was investigated using a 200 milligram CLEAN SCREEN™ EXTRACTION COLUMN (See Appendix One).

In the extraction experiments, fortified hair (negative hair soaked overnight in a drug/methanol solution) was analyzed to determine which extraction technique provided the best recovery of a drug from the hair when compared to non-processed controls, based

upon EMIT rates and GC/MS mass ratios. The liquid extraction utilizing n-butyl chloride provided the cleanest extraction for all of the drugs analyzed, as determined by the total mass ratio (total area for all peaks in the chromatogram) and number of extraneous peaks (noise) identified using the GC/MS.

A. pH Effecting the Extraction Efficiency

The development of a general analytical method required the inclusion of a large variety of drugs into one digestion-extraction procedure. Based upon the first set of experiments regarding the digestion solvent, a basic solvent was best to solublize the hair fiber. This alkaline pH could make the extraction solvent less efficient in pulling the drugs from the digestion solution. To explore this issue, an experiment was done using five hair samples weighing approximately 10 milligrams each. These samples were fortified with the following drugs solublized in methanol: amphetamine, methamphetamine, chloropheniramine, lidocaine, meperidine, codeine, oxycodone, amitriptyline, caffeine, and the internal standards benzphetamine and papaverine. The five samples were fortified at equivalent concentrations for a period of 24 hours (overnight). A negative control, with internal standards, and a positive fortified control were analyzed parallel to the five samples and served as quality controls. The five samples were digested in 1 N sodium hydroxide for one hour with constant stirring in a hot water bath of 60 ° Celsius. Samples were allowed to cool to room temperature and the pH was adjusted to 6, 8, 9, 10, and 13 using 1 N HCl, the negative and positive controls were kept at a constant pH 13. The samples, including the negative and positive controls, were extracted and analyzed as described in Appendix One. The "cleanest" extraction, based upon EMIT

rates and baseline noise observed on the GC/MS was achieved using the n-butyl chloride at a basic pH 13.

B. Volatility

A difficultly involving the detection of amphetamine-like drugs in fortified hair using our preliminary screen with the EMIT was observed. This prompted exploration into the volatile nature of amphetamine and the effect of digestion temperature on drugs such as amphetamine and amphetamine analogs. Amphetamine slowly volatilizes at room temperature. Volatilization becomes negligible if silanized glass test tubes are used and the tubes are capped (allowing the tubes to cool to room temperature before opening).

To determine how the increased temperature of the digestion bath was affecting the volatile nature of amphetamine like compounds, triplicate sets of three positive hair samples were fortified overnight using drug solutions, as described in the preceding section. The digestion solvent was 1 N sodium hydroxide. Each of the three samples was placed in water baths of 30, 45, and 60 degrees Celsius for 30, 60, or 120 minutes respectively. One sample was left in the 30-degree water bath for more than 180 minutes. The samples were then extracted and analyzed as described in Appendix One. An inverse relationship between water bath temperature and length of digestion time was observed. We were able to detect amphetamine and amphetamine-like compounds at the lower temperatures (>40 degrees Celsius). However, longer digestion time was required to achieve solubilization of the hair fibers, based upon visual observation.

V. Analytical Procedures

Throughout the history of forensic hair analysis, many instrumental methods have

been employed to identify drugs, including radioimmunoassay, high-performance liquid chromatography, and gas chromatography-mass spectrometry(GC-MS). One type of presumptive screen utilized for determining the presence of drugs of abuse in biological samples is the EMIT. The EMIT assays provide qualitative analysis of blood and urine specimens for the presence of the following classes of drugs of abuse: amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, opiates, and propoxyphene. The assays utilize a homogeneous enzyme immunoassay technique. Samples are mixed with two reagents:

1.) Reagent A contains antibodies to a particular drug, the coenzyme nicotinamide adenine dinucleotide (NAD), and a substrate for the enzyme glucose-6-phosphate dehydrogenase (G6P-DH).

2.) Reagent B contains a drug derivative labeled with the G6P-DH enzyme. When reagents are added to the sample, the antibody binds to the drugs it recognizes, and the enzyme-labeled drug combines with the remaining antibody binding sites, decreasing the enzyme activity. Some enzyme remains unbound and, therefore, stays active in the reaction mixture. This residual enzyme activity is directly related to the concentration of the drug in the sample. The active enzyme converts NAD to NADH, resulting in a photometric absorbance change. By comparing the absorbance change for a sample with that of the cutoff calibrator that contains a known quantity of drug metabolite, drug presence in the sample can be determined. Three calibrator levels are provided for each assay: negative (drug free calibrator), cutoff (the cutoff, or low calibrator), and high (the positive calibrator). The reaction rate of the cutoff calibrator serves as the reference point

for determining samples results. A sample is considered positive if its reaction rate is equal to or greater than that of the cutoff calibrator. Negative and positive calibrators are analyzed as controls to validate reagent and instrument performance. Each assay takes approximately 1 minute to complete. The variety of assays coupled with the brief analysis time made this instrument ideal for a presumptive screen for hair analysis.

While there are a variety of presumptive screens which are acceptable for drug analysis, currently, the most reliable, sensitive, and specific method of analysis used to *confirm* the presence of drugs in hair is gas chromatography-mass spectrometry (GC-MS). (29) The underlying chemical and physical properties of the drug such as pka, solubility, and functional groups determine which instrumental parameters are selected to afford better separation and identification of the drugs of interest. Fragmentation patterns of xenobites are obtained through either one of two ionization modes, electron ionization (EI), or chemical ionization (CI). In positive-ion EI, positively charged sample ions are formed when electrons from the filament ionize sample molecules as follows:

$$(S) + (e-) = (S+) + (2e-)$$

The energetic electron that interacts with the sample molecule often deposits more energy in the sample molecule than is needed for ionization alone, therefore, the sample ion, S+, often decomposes through a complex series of unimolecular decomposition, with charged and neutral fragments. The MS detector determines the masses and abundances of the various ions present, and a plot of this information is the familiar positive-ion EI mass spectrum. The extensive fragmentation that is produced during EI is useful as a fingerprint which can be used for identification of the compound. Positive-ion EI mass spectra are the

most common type of mass spectra, and identification can often be achieved through a computer search of an extensive library of EI spectra, such as the NIST library. The conditions for obtaining positive-ion EI spectra are well standardized. Therefore, positive-ion EI was our method of choice.

A. Peak Criteria

Three criteria were utilized to determine the appearance of "peaks" on the GC-MS. First, the signal-to-noise ratio (over the background) has to exceed the **mean** area of six random data points (three from each side of the peak in question) **plus three standard deviations**. Second, the retention time of the compound of interest should match the retention time of extracted standards of commercially prepared quality control samples (Biorad or Utak), or a prepared bovine whole blood sample fortified with primary standards, or a mixture of primary standards prepared in methanol (or other suitable standards). The retention time must be within +/- 3% of the aforementioned standards. In addition to the criteria mentioned above, the third criteria involves obtaining a match quality (reported by the instrument) which exceeds 850 counts for full scan EI data.

VI. Sample Size

In forensic settings, one of the key issues in any analysis is availability of a sample. This becomes exceedingly important in analysis of hair samples obtained from deceased individuals whose remains have been returned to the family. Sample size becomes a factor if the quantity of sample is limited and multiple analyses are needed for presumptive screening and confirmation purposes. To use available hair samples most efficiently the smallest sample possible was analyzed in order to conserve the finite

supply of hair yet still be above the limit of detection of the analytical method; also, the presumptive screen and confirmation was performed on the same extract. To address the issue of sample size we fortified four hair samples weighing 5, 10, 25, and 50 milligrams with equivalent drug concentrations (in nanogram drug/milligram hair). The digestion, extraction, and analytical procedures as described in Appendix One were followed. The minimum samples size of hair that provided reproducible results and detectable quantities of drugs was 10 milligrams. This sample size coincides well with values found in the literature. (30, 29, 31) Presumptive positive and confirmatory analysis were preformed on the same extract in order to conserve the initial supply of sample. The hair supernatant is split after the final reconstitution (350 micro-liters methanol) into two samples: 300 micro-liters of extract is pipetted into an EMIT plastic sample cup and 50 micro-liters is pipetted into an auto-sampler vial for analysis on the GC/MS. The advantages of splitting the sample are twofold: first, presumptive and confirmatory screens are run using one extract:;second, we bypass evaporation and decontamination effects (from the plastic EMIT sample cup) afforded by the first screen.

Chapter 3. Materials and Methods

I. Analytical reagents

HPLC grade reagents were obtained from Fisher Scientific; saturated borate solution (pH 9.2) was prepared in distilled water; 1 N hydrochloric acid and 5 N sodium hydroxide were prepared in distilled water. Drugs used to fortify negative hair were purchased from Fisher Scientific. A 1 milligram/milliliter stock solution of benzphetamine was prepared in methanol to serve as the internal standard. Drug standards (multiple drug solutions prepared in methanol), were also prepared 1 milligram/milliliter concentration and served as positive controls. Working solutions were prepared by dilution of the stock solution in methanol to 10 nanogram/microliter concentrations. Drug solutions were stored at -20 degrees Celsius until use. Hair samples (fortified and case samples) were stored at ambient temperature until use.

II. Hair preparation.

Negative control hair samples were collected from several drug free volunteers (determined by urine analysis) throughout the length of the project. Both individuals were female with brown hair. Hair samples were cut from distal portions of the hair fiber. Negative samples were cut into 1 centimeter sections, and 10.0 (+/- 0.1) milligrams of the sectioned hair was used as a control. Negative controls were fortified with 250 nanograms of an internal standard, benzphetamine. Hair samples were allowed to fortify for 24 hours to allow the drug to sufficiently adhere to the hair fiber. Upon conclusion of the 24 hours, the fortified samples were placed under a gentle stream of nitrogen to evaporate off the drug solution. Positive controls were prepared in a similar manner as negative controls.

However, in addition to 250 nanograms internal standard, 2000 nanograms of the following drugs were added to the hair sample: amphetamine, methamphetamine, meperidine, oxycodone, codeine, amitriptyline, lidocaine, caffeine, and papaverine. Fortified hair samples were not washed prior to digestion to ensure that 100 percent of the internal standard and grob drugs (drugs used to fortify positive controls) were available for digestion and extraction. (36) Non-fortified positive samples (i.e., case samples) were collected from deceased individuals across the state of Montana. Samples were pulled from the subject's scalp in the posterior vertex region of the head. If head hair was not available, pubic and axilliary hair was collected. The samples were sent to the Montana State Crime Laboratory in hair collection kits which were prepared at the crime lab and distributed to county coroners during preliminary stages of this research project. Case samples were stored at ambient temperatures in the collection kits. Each sample was washed as described in Appendix One. The sample size of the case sample was equal to that of the control samples. Case samples were spiked with 200 nanograms of internal standard just prior to the extraction step.

III. *EMIT Analysis*

The presumptive screen is performed on a SYVA™ ETS™ Plus System. The photometer uses a tungsten-halogen 14 volt bulb as the light source with an operating range of 0.000-2.000 absorbance units and wavelength of 340 (+/- 2) nanometers. Each analysis uses 17.5 micro-liters of reagent A and reagent B, and 5.0 micro-liters of the hair supernatant sample. The accuracy of the instrument is +/- 2.0 % of the sampled volume, and +/- 2.5 micro-liters +/- 2.0% of dispensed volume. The throughput of the instrument

is approximately 73 tests per hour. Data output is expressed using a thermal dot matrix printer. The external computer is a RS-232C with switch selectable baud rate.

IV. *Chromatography.*

The confirmatory analysis is performed on a Finnigan MAT™ GCQ ion trap mass spectrometer, with a A200S autosampler. The injection port temperature is maintained at 230 ° Celsius. Separation is achieved on a silicon capillary column (30 m x 0.25 mm i.d., J & W DB5MS). Electrons are emitted by a filament in the ion source and are accelerated to a kinetic energy of approximately 70 electron volts. Transfer line temperature is 200 ° Celsius and ion source temperature is 230 ° Celsius. The GC temperature program is as follows: initial temperature, 80 ° Celsius, hold 1.00 minutes(s); 30 ° Celsius/minute increase until 200 ° Celsius; 10 ° Celsius/ minute increase until 300 ° Celsius; hold for 5 minutes; total run time, 20 minutes. The scan mode is as follows: begin scan at 3 minutes; full scan mode; mass 40-400 atomic mass units. The GCQMS detector utilizes an ion trap mass analyzer with an ion source external to the mass analyzer.

Chapter 4. Analysis of Non-fortified Samples (Pilot Case)

I. Pilot Case

To determine if the digestion-extraction procedure designed using fortified hair would extract drugs in non-fortified (positive) hair, a sample of hair collected by the state pathologist from a deceased person in a forensic case was analyzed. The case history of the decedent who donated the hair was reviewed prior to analysis to ensure that the individual participated in drug use, therefore the hair sample had high probability of containing endogenous drug.

All cases analyzed following the pilot case would be performed in a blind study. The case history for all samples would not be known to the investigator until after the conclusion of the hair analysis. After the hair was analyzed, the case history of the individual, including the results of the toxicological analysis, was reviewed for comparison purposes.)

A. Case History

A deceased 44 year-old white male was admitted to a local hospital in early October.. The decedent was discovered in a local park and was listed as an apparent dumped body by the county coroner. The body was beginning to undergo decomposition processes. A urine specimen was drawn by the hospital staff and tested for drugs of abuse using an ELISA (immunoassay) method. The specimen tested positive for cocaine metabolites and opiates. The subject was relocated to the Montana State Crime Laboratory for further investigation.

B. Analysis of Toxicological Specimens

The next day an autopsy was performed by the state pathologist and additional toxicological specimens (blood, urine, vitreous humor, hair, and the following tissues: liver, kidney, and brain) were collected for analysis. Urine spot test for salicylates, phenothiazenes, imipramine, and placidyl were unremarkable for the decedent. Samples run on the EMIT revealed an unremarkable blood specimen while the urine specimen was positive for cocaine metabolites and opiates, and had an increased rate for amphetamine/amphetamine-like analogs. Basic extraction of the urine specimen (analyzed on the GC-Nitrogen Phosphorous Detector and confirmed on the GC/MS) confirmed the presence of methamphetamine, nicotine, cotinine, carisoprodol, cocaine, hydocodone, morphine, codeine and fentanyl. Acidic/neutral extraction of the blood (analyzed and confirmed on the GC/MS) confirmed the presence of meprobamate, carisoprodol, and fentanyl. Analysis of the blood for alcohol by headspace autosampler was unremarkable. Approximately two months after the autopsy was performed and the biological fluids were analyzed, two samples of the decedents hair, weighing 20 and 60 milligrams, were analyzed.

C. Analysis of Hair

The hair sample was cut into 1 centimeter lengths at the most proximal portion of the hair shaft. Decomposition of the body and skin slippage did not allow the hair to be pulled cleanly from the scalp. Therefore, skin cells were removed from the hair sample with small tweezers until visual inspection verified a clean hair sample. The hair was weighed into 20 and 60 milligram specimens and these two samples were washed as described in Appendix One. The wash of each sample was analyzed parallel to each hair

sample on the EMIT and GC/MS. Digestion and extraction procedures as described in Appendix One were followed to prepare the hair sample for instrumental analysis. Quality controls (positive and negative) were analyzed parallel to the case sample to evaluate instrumental performance (precision and accuracy) for the EMIT and GC/MS.

EMIT analysis of the wash was unremarkable for both specimens (20 and 60 milligram samples). The larger hair specimen (60 milligrams) tested positive for amphetamine/amphetamine-like compounds, and resulted in increased rates for cocaine, barbiturates, benzodiazepines, and opiates. The smaller hair specimen (20 milligrams) tested positive for amphetamine/amphetamine-like compounds.

GC/MS analysis of the wash for both specimens was unremarkable. GC/MS analysis of the hair specimens revealed a much cleaner spectrum for the smaller sample versus the larger sample. Although the larger sample tested positive for more assays on the EMIT, more contaminants were apparent on the GC/MS (as identified by the appearance of many more extraneous peaks resulting in a noisy baseline). Nicotine, phenethylamine (a by-product of decomposition) and the internal standard, benzphetamine were confirmed. In the smaller sample, nicotine, phenethylamine, and benzphetamine were also confirmed. This sample was much cleaner than the larger sample, and mass addition revealed the molecular ions for codeine, although the presence of this drug could not be confirmed.

Two hair samples (20 milligrams each) were prepared (and washed) as described above. After the initial digestion step, one of the samples was extracted using a standard benzoglecgonine (primary metabolite for cocaine) extraction procedure and the other

sample was extracted using a standard acidic/neutral procedure (Appendix One). Both of these samples were analyzed using GC/MS. The presence of cocaine or cocaine metabolites could not be confirmed. However, analysis of the acidic/neutral extract confirmed the presence of phenobarbital in the hair.

D. Discussion

The profile of the hair analysis may be explained in part by the following:

1.) Generally, parent compounds are found much more readily in hair versus their metabolites. (37)

2.) Of the 10 drugs found in the blood (3) and urine (9), cotinine, meprobamate, and codeine are all metabolites.

Interpretation of the results must be made with caution. The decedent's interest in drugs of abuse is supported by the fact that phenobarbital was discovered in the hair sample. In addition, the non-scheduled drug nicotine was also confirmed in the hair sample. The wash extracts were unremarkable for either of the two drugs confirmed in the decedents hair, indicating that the drugs were from endogenous origin. Interestingly, a by-product of decomposition (phenethylamine) was confirmed in the hair sample, however, there was no trace of the contaminant in the wash extract. This indicates that the contaminant was deposited within the hair shaft itself, after death.

In summary, the initial digestion-extraction procedure confirmed the presence of one drug and led investigators to further analysis which ultimately confirmed the presence of a second drug of abuse. These findings indicate the procedure designed using fortified positive hair samples can extract non-fortified positive hair samples (endogenous drug)

and lead to the qualitative determination of drugs of abuse in hair.

E. Reproducibility

To further ensure instrumental integrity, a different case was submitted to duplicate analysis. This experiment was designed to test the reproducibility of the analytical procedure (by analyzing the same extract in repeat series within a twenty-four hour time period), and to test the reproducibility of the methodology (by splitting one hair sample and running parallel digestion/extraction). In other words, we wanted to identify and confirm the same drugs each time we submitted the hair sample to our analytical and bench methods. The decedent was a male subject with dark brown/ black hair. Analysis of decedent's urine specimen revealed the presence of acetaminophen, meprobamate, caffeine, carisoprodol, propoxyphene, norpropoxyphene, amitriptyline, and nortriptyline. Analysis of the decedent's blood tested positive for the following compounds: caffeine, propoxyphene, norpropoxyphene, acetaminophen, ibuprofen, carisoprodol, and meprobamate. A 20 milligram hair sample was split into two portions and analyzed separately, following the wash and digestion/extraction procedures described in Appendix One. Results for both fractions of the split hair sample resulted in the following confirmations: nicotine, meprobamate, carisoprodol, propoxyphene, norpropoxyphene, and amitriptyline, see Appendix Three . Analysis of the wash (for both fractions) revealed the presence of carisoprodol and propoxyphene. The presence of compounds in the wash promotes cautious interpretation of the hair results. Since the other five compounds identified in the hair samples did not appear in the wash, it might be infered that the two compounds in the wash were extracted out of the hair by the water. It is interesting to note

that the presence of two metabolites (meprobamate and norpropoxyphene) were identified in the hair shaft. Generally, the analysis of hair for metabolites of parent compounds present a difficult problem for forensic scientists (see Chapters 4 and 5 for discussion of metabolites in hair).

To strengthen the validity of instrumental analysis, two hours after the first two samples were analyzed, one of the samples was analyzed again. The second analysis confirmed the presence of all compounds present in the first analysis, see Appendix Three.

The combination of the reproducibility experiments for both the analytical procedure and bench methodology strengthen the effectiveness of the detection protocol.

Chapter 5. Results of Hair Analysis for 29 Case Samples

I. Results

Hair samples were collected by county coroners and the state pathologist throughout a time period of 22 months. Samples were stored in their original collection kits at ambient temperature until analysis. Upon analysis, cases were randomly grouped into sets of five and analyzed in parallel with fortified positive and negative hair standards, and a positive drug control. Each case sample was washed according to the procedure described in Appendix One.

The results of 29 case studies are summarized in Table 2. In addition to the hair analysis and toxicological results, gender, age, hair color, and submitting agency were recorded. Seven female and 22 male subjects participated in the study.

Table 2. Summary of results for 29 cases submitted for hair analysis.

Case Number	Agency	Gender	Hair Color	Drugs in Urine	Drugs in Blood	Drugs in Hair	Drugs in Wash
15-T	Benefis West	M	Dark Brown	none detected	none detected	nicotine	none detected
983355	Benefis West	F	Dark Brown	N/A	(vitreous) none detected	none detected	none detected
122298	Benefis West	M	Brown	none detected	none detected	none detected	none detected
988037	Hill Co. Sheriff	M	Brown	none detected	none detected	none detected	none detected
982465	Missoula Co. Sheriff	M	Brown Gray	N/A	ethanol caffeine nicotine lidocaine	nicotine hydro-codone	none detected
983358	Benefis West	M	Brown	N/A	ethanol ibuprofin	nicotine aspirin	none detected

Case Number	Agency	Gender	Hair Color	Drugs in Urine	Drugs in Blood	Drugs in Hair	Drugs in Wash
983538	Benefis West	M	White	nicotine caffeine	none detected	aspirin	none detected
990056	Benefis West	M	Black	caffeine diphen-hydramine	caffeine	aspirin	none detected
983970	Benefis West	M	Gray	none detected	none detected	nicotine	none detected
983415		F	Blond	No Suitable Sample	No Suitable Sample	nicotine aspirin	none detected
981387	Missoula Co. Sheriff	M	Dark Brown	ephedrine pseudo-ephedrine nicotine cotinine caffeine	ethanol caffeine	nicotine ephedrine	none detected
981426		F	Brown	methadone pro-methazine codeine diazepam nordiazepam hydrocodone hydroxine paroxetine	nicotine caffeine ibuprofen aspirin meprobamate carisoprodol	nicotine ephedrine phen-iramine hydro-codone norpro-poxyamide	none detected
981691	Madison Co. Coroner	F	Black	N/A	ethanol nicotine cotinine caffeine	nicotine diphen-hydrinate	diphen-hydrinate
981329	Lake Co. Coroner	F	Dark Brown	amphet-amine methamphet-amine cannabinoid	methamphet-amine	methamphe t-amine ephedrine	none detected
981743	Ravalli Co. Coroner	M	Light Brown	N/A	ethanol nicotine	nicotine lidocaine hydrocodone	none detected
983636	Richland Co. Coroner	M	Brown with White	N/A	none detected	nicotine	none detected

Case Number	Agency	Gender	Hair Color	Drugs in Urine	Drugs in Blood	Drugs in Hair	Drugs in Wash
982301	Beaverhead Co. Coroner	M	Light Brown	no suitable samples	no suitable samples	nicotine phenethy l-amine propyl-phenethy l-amine	none detected
980735	Ravalli Co. Coroner	M	Brown	nicotine cotinine cannabiniod	ethanol caffeine cannabinoid	nicotine	none detected
983356	Toole Co. Coroner	M	Dark Brown	ephedrine psuedo-ephedrine nicotine cotinine caffeine	ethanol	nicotine	none detected
983357	Cascade Co. Coroner	M	Dark Brown	nicotine cotinine mepro-bamate carisoprodol amitrip-tyline norami-triptyline oxycodone cannabinoid	nicotine amitriptyline norami-triptyline diazepam nor-diazepam oxycodone carisoprodol mepro-bamate	nicotine amitrip-tyline oxycodone carisopro-dol	none detected
980819	Lake Co. Coroner	M	Brown	caffeine	caffeine lidocaine	nicotine	none detected
980103	Gallatin Co. Coroner	F	Black	N/A	ethanol	nicotine	none detected
983372	Benefis West	F	Black	N/A	caffeine	nicotine	none detected
980890	Ravalli Co. Coroner	M	Brown	N/A	ethanol diphen-hydramine propoxy-phene norpro-poxyphene amitriptyline norami-triptyline	nicotine diphen-hydramine amitrip-tyline	none detected

Case Number	Agency	Gender	Hair Color	Drugs in Urine	Drugs in Blood	Drugs in Hair	Drugs in Wash
980609	Flathead Co. Coroner	M	Brown	nicotine caffeine	ethanol nicotine caffeine	nicotine	none detected
983582	Cascade Co. Coroner	M	White	caffeine aspirin	caffeine	none detected	none detected

In the population of case samples, 38 confirmations occurred in urine, 35 confirmations in occurred blood, and 40 confirmations in the hair samples. The above cases represent diverse parameters (including gender, age, environment, time of collection after death, and cause of death). These parameters, combined with the relatively small number of samples, precludes broad conclusions. However, several trends are observed in the data obtained from the experiment:

1.) In eight cases metabolites of active compounds were identified in the blood or urine and did not appear in the hair. While metabolites for methadone, propoxyphene, morphine, marijuana, PCP, cocaine, and tricyclic antidepressants have been shown to bind to hair, to achieve detection at the lower drug concentrations, derivatization of the hair samples is often required. (28,38-42) For some drugs such as amphetamine, PCP, and marijuana, the metabolites are either in extremely low concentration in the hair (relative to the parent compound) or non-detectable.(38) Our data supports these observations.

2.) A variety of drugs which were not identified in the other biological samples (25 in blood, and 10 in the urine) were confirmed in the hair. These findings support the role of hair analysis as a useful supplemental screen in toxicological analysis. A more complete drug history may be afforded when hair analysis is combined with the traditional

forensic toxicology protocol. In addition, ten confirmations of drug use initially identified in the blood were observed in the hair, and eleven confirmation of drug use identified in the urine were supported by hair analysis.

3.) Drugs were identified 40 times in the hair samples. However, only one of the wash samples tested positive for drugs. The drug confirmed in the wash of case 98-1691 was diphenhydrinate (Dramamine). This correlates to a 2.50% extraction efficiency of the wash solvent (as defined by number of drugs extracted in the wash divided by total number of drugs extracted in the hair sample).

4.) Using the digestion-extraction protocol described in Appendix One, drugs belonging to the benzodiazepine drug class were not detectable. Diazepam, a prototypical benzodiazepine, has a pKa of approximately 3.4, which is much lower than that of drugs classified in the other six drug classes. The absence of the benzodiazepines in the hair samples could be the result of an extreme pH difference between our digestion-extraction media and the drug.

5.) Three cases (983355, 122298, and 988037) tested negative for all three specimens analyzed. If the detection protocol is limited to drugs of abuse, 14 of the 26 cases tested negative for drugs in the blood and urine specimens. The 14 corresponding hair samples also tested negative for drugs of abuse.

Chapter 6. Conclusions

I. Discussion

A. Drug Structure

The difference in appearance of drug in the blood, urine, and hair specimens can be explained in part by structural parameters of the drugs (lipophilicity, pKa) and different pharmacokinetic behavior (half-life, metabolism, protein binding, and pH). (18) For example, the half-life and metabolism rate of a drug relate directly to the duration of exposure to hair binding sites. Protein binding and pH of a drug effect possible competing reactions with hair binding sites and overall incorporation into the hair fiber (see Figure 7). In several cases, a qualitative trend is seen between drugs identified in all samples (983355,122299, 988037, 981426, and 983357), however, there appears to be a very strong deviation for some of the subjects (983358, 983356, and 980890). This same phenomena was reported in the literature for therapeutic drugs as well as illicit drugs of abuse. (43)

B. Pigmentation

Most (69%) of the individuals included in this study had brown or black hair. As detailed earlier (Chapter 1, Section III), color is determined by the quantity of pheomelanin and eumelanin present in hair. Black and brown hair contain more eumelanin than blond or white hair. Unfortunately, there are few studies on the effect of hair color on the incorporation of drugs of abuse into human hair. Several laboratories have reported that hair color is responsible for the greater accumulation of haloperidol and ofloxacin in black hair than in white hair. (44) Other investigators have demonstrated that

drugs of abuse bind to the hair of albino animals in amounts that are similar to those found in pigmented animals. (45) These findings suggest that drugs need not be incorporated into the melanin pigment. This paper reports the finding of nicotine and acetaminophen in 2 samples of white/blond hair. The small sample population precludes any generalizations from the data presented here regarding pigmentation and the binding of drugs to hair.

C. Individual Cases

1. Interestingly, several cases present unique information regarding the presence of nicotine in the decedents hair samples. In 15-T, 983358, 983970, 983356, and 980819 nicotine was not identified in either the blood or urine, yet it was confirmed in the decedent's hair. One hypothesis might be that these subjects did not ingest the compound, that the drug was a contaminant on the hair fiber (exogenously deposited from the environment). However, nicotine was not identified in the wash extracts for any of the hair samples. It can be inferred that the compound was endogenous, and had been cleared from the circulatory system prior to death.

2. Two cases in particular reinforce the need for hair analysis as a supplement to traditional methods. In these cases (98-2301, 98-3415), no suitable samples were obtained in the toxicological collection kits. Due to decomposition, analysis of the biological fluids was futile. Analysis of the hair samples revealed the presence of nicotine and acetaminophen, in addition to numerous products of decomposition. While nicotine and

acetaminophen are not drugs of abuse, they offer further evidence to support the usefulness and applicability of hair analysis in forensic screening.

3. This procedure was particularly sensitive for opioids, within the limitations of drugs detected by our analytical methods. With only one exception (when codeine was identified in the biological fluid and not in the hair), all opioids were confirmed in the hair if identified in other biological samples .

4. The presence of lidocaine in decedent's blood or urine samples represents several interesting possibilities. Lidocaine is a local anesthetic commonly administered to patients in crisis situations upon arrival to hospital facilities. In 982465 and 980819 lidocaine was confirmed in the blood or urine but was not identified in the hair sample. Two explanations for this discrepancy are:

   - lidocaine does not extract well using the protocol and was present in the hair, but was not detected by the bench/analytical methods (or lidocaine does not fully integrate the hair fiber);

   - lidocaine was administered at an emergency facility immediately prior to the death of the individual, but the time interval to death was so short the drug did not penetrate the hair follicle of the individual.

While the hospital records for the two decedents involved in the above cases are not yet available to this investigator, the identification of lidocaine in two additional, separate hair samples has shown that this compound does bind to the

hair shaft and will be extracted by the procedure. Further investigation is needed to determine if the appearance of lidocaine in 982465 and 980819 is a result of emergency treatment.

5. In case 122298, all three samples (blood, urine, and hair) tested negative for the presence of drugs for the seven drug/drug class assays. This case serves as an excellent "negative control" for our research design. Fourteen cases were negative for drugs of abuse (or prescription drugs) analyzing the blood or urine, and all fourteen hair samples were also negative. These findings need to be interpreted with care. At this point in the science of hair analysis, there is not sufficient data to support the statement that a negative hair sample, without additional information from blood or urine samples, indicates that the individual is a drug free. Interpretation of negative, or drug free hair samples, gives information of drug history at the point in time designated by the length of hair (using an average growth rate of one centimeter of hair per month). In other words, one month prior to death (as in our research protocol) the individual was drug free, within the limitations of our experimental design.

II. Summary

One of the greatest challenges in the area of hair analysis has been the development of sensitive MS technologies to provide reliable confirmation of drugs in toxicological analysis. This paper describes a novel approach to the confirmation of endogenous compounds from seven different drug classes using one general, comprehensive digestion-

extraction procedure. Instrumental techniques, hair collection protocol, and sample preparation described in this paper stand as testimony to the evolution of criteria for confirmation of drug presence that are generally recognized as scientifically sound. Hair analysis proves to be a very useful tool for qualitative retrospective evidence of drug intake within a wide time window. The results described in this report further current understanding of the general parameters of drug disposition in hair. In order to get more detailed information about the timetable of drug consumption, drug binding kinetics, and quantitative aspects of drugs in hair, much more experimental effort is needed to exhaust the possibilities of the method, which are more or less dictated by individual cases. The relationship between new and traditional approaches to forensic analysis should not be viewed as one of competition between blood, urine, and hair analysis, but rather as one wherein the different techniques are in synergistic interaction in solving the unique forensic challenges associated with the identification and timing of drug use. In this respect, the particular value of hair analysis lies in the ability to determine long-term usage of drugs of abuse rather than the short-term elimination provided by blood and urine.

Chapter 7. Decontamination Revisited

I. Endogenous versus Exogenous Drug Binding

One key issue regarding drug analysis in forensic cases is the potential for identification of drugs that have been bound exogenously, rather than via ingestion. Decontamination steps are taken (washing the hair sample) prior to analysis of the hair sample to ensure that the environmental contribution (from vapors, smoke, or external residue) is minimized. Lack of availability of good endogenous samples to compare with exogenous samples makes validation of any wash protocol difficult. One way to circumvent this issue, and remove the possibility of extracting the endogenous drug from the hair shaft, is to determine the location of the drug on, or in, the hair shaft.

A novel approach to differentiate between endogenous and exogenous drug exposure was explored in one of the experiments designed using a rat model. In this experiment, the lower dorsal region of three Sprague-Dawley rats were shaved on days 0 (the negative control), 7, and 21. In addition to the above samples, a positive control was prepared using negative rat hair and soaking the rat hair in a solution of codeine dissolved in methanol. Each rat was injected with 20 milligram codeine/ killogram animal on day 0, 7, and 14. All samples underwent the same wash procedure described in Appendix One. Upon conclusion of the decontamination procedure and a one day drying period, all hair samples were embedded in paraffin , sectioned using a microtome, and stained using the monoclonal antibody protocol described in Appendix Two.

The negative control was characterized by the lack of antibody binding to the hair section through microscopic visual observation, Figure 8 (A). The positive control did

have apparent antibody binding sites on the exterior, or outside of the hair fibers, Figure 8 (B). These binding sites were displayed as dark brown domains on the exterior surfaces of the hair fiber. The hair samples collected on day 21 from the rat's lower back, which contained endogenous drug, differed from the positive and negative controls. The endogenous samples were stained on the inner portions of the hair shaft, towards the medulla and cortex of the hair fibril, Figure 8 (C). These findings are supported by data obtained in other research facilities analyzing horizontal sectioning of hair shafts using infrared microscopy. (46)

To expand upon the findings in the above study, a similar protocol was applied to a human model. In this experiment, 10 milligrams of positive hair (250 nanograms hydrocodone solublized in methanol fortified onto the hair fibril for 8 hours, then evaporated to dryness) and 10 milligrams negative hair standards were placed in a drying oven (70° Celsius) overnight. This drying step was incorporated to replace the "fixing", or dehydrating step in the rat model. The rationale for the drying step was based upon the discovery of wash solvents extracting the drugs from hair samples. By avoiding unnecessary solvent interaction, an attempt to avoid extracting the drug from our hair sample into the fixing reagents was explored. The positive and negative controls were embedded in paraffin, sectioned (at 5 microns), mounted, and immunocytochemistry was performed as described in Appendix Two. In addition to the controls, two cases which tested positive for opioids, (981426 and 981743; hydrocodone) were also analyzed.

II. Results

The human hair controls provided much differentiation between the staining

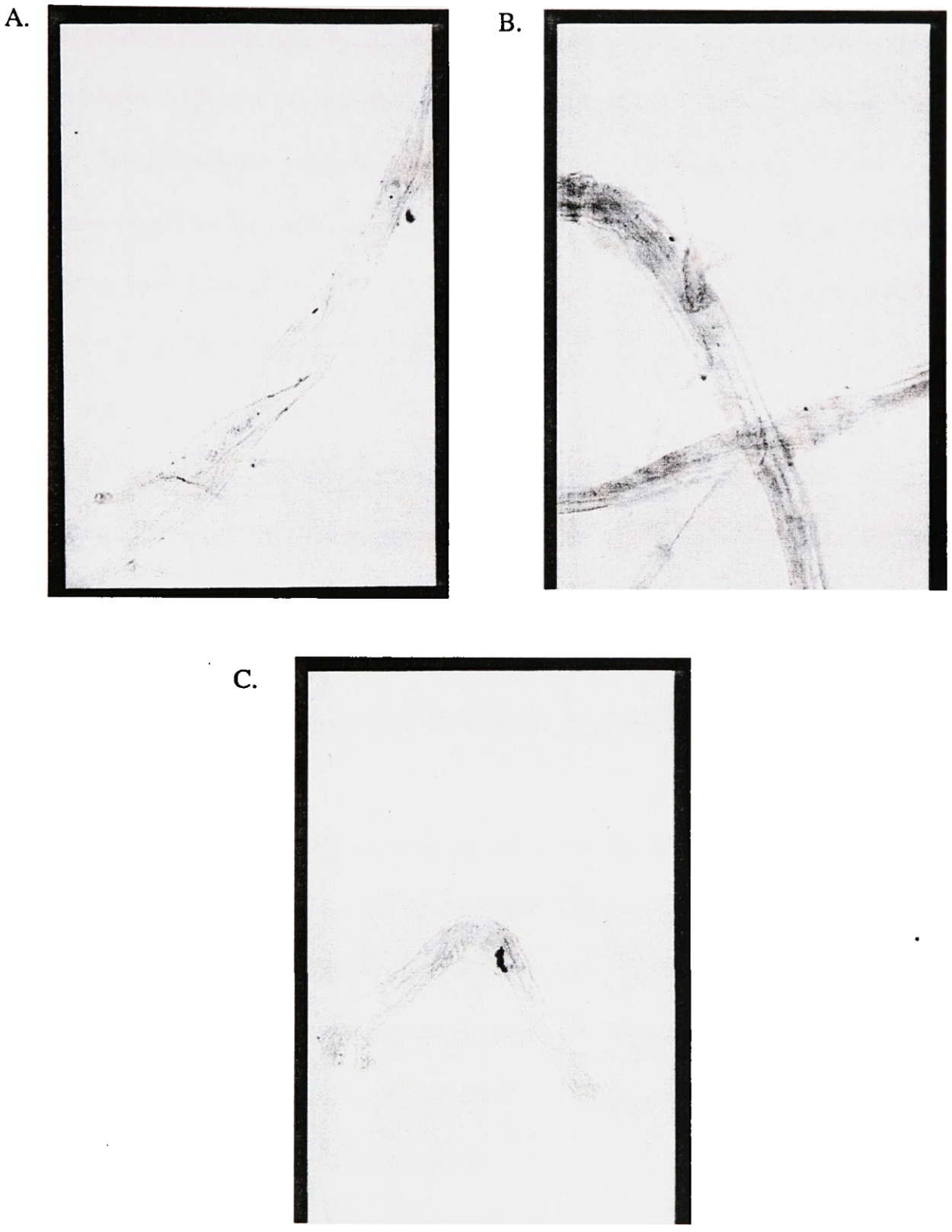


Figure 8: Photographs of (A) Negative rat hair, (B) Fortified positive rat hair; codeine, and (C) Non-fortified positive rat hair (Staining with DAB)

patterns of drug versus non-drug controls, Figure 9. The negative (or drug-free) hair sample, Figure 9 (A) has little "background" staining compared to the fortified positive control, Figure 9 (B). All of the staining in the positive control is exterior to the cuticle, with very little stain penetrating the cortex. It is interesting to note that the two hair specimens chosen for the endogenous (case) study appear to bind hydrocodone in different areas of the inner hair shaft. Case sample 981743, Figure 9 (C) is stained in the medulla region of the hair fiber, whereas 981426, Figure 9 (D), a hair sample without a medulla, is stained in the outer cortex region of the hair shaft.

It is important to note that no histochemical work involving hair analysis is available to this author for comparison and this work serves only to initiate a preliminary understanding of the possibilities available through immunocytochemistry for the differentiation of endogenous versus exogenous drug binding. Further research is required to determine is this approach is viable for additional drugs of abuse.

III. Discussion

This work offers exciting evidence for a new detection method of endogenous versus exogenous drug deposition. Within a limited human model, differentiation between localization of endogenous and exogenous hydrocodone has been observed. The application of immunocytochemistry to other drugs and hair types has not been explored. While further research is need to expound upon these preliminary data, the significance of discrimination between "clean" and contaminated hair samples in forensic cases can be expected to escalate as the science of drug analysis in hair becomes more proficient.

A.

B.

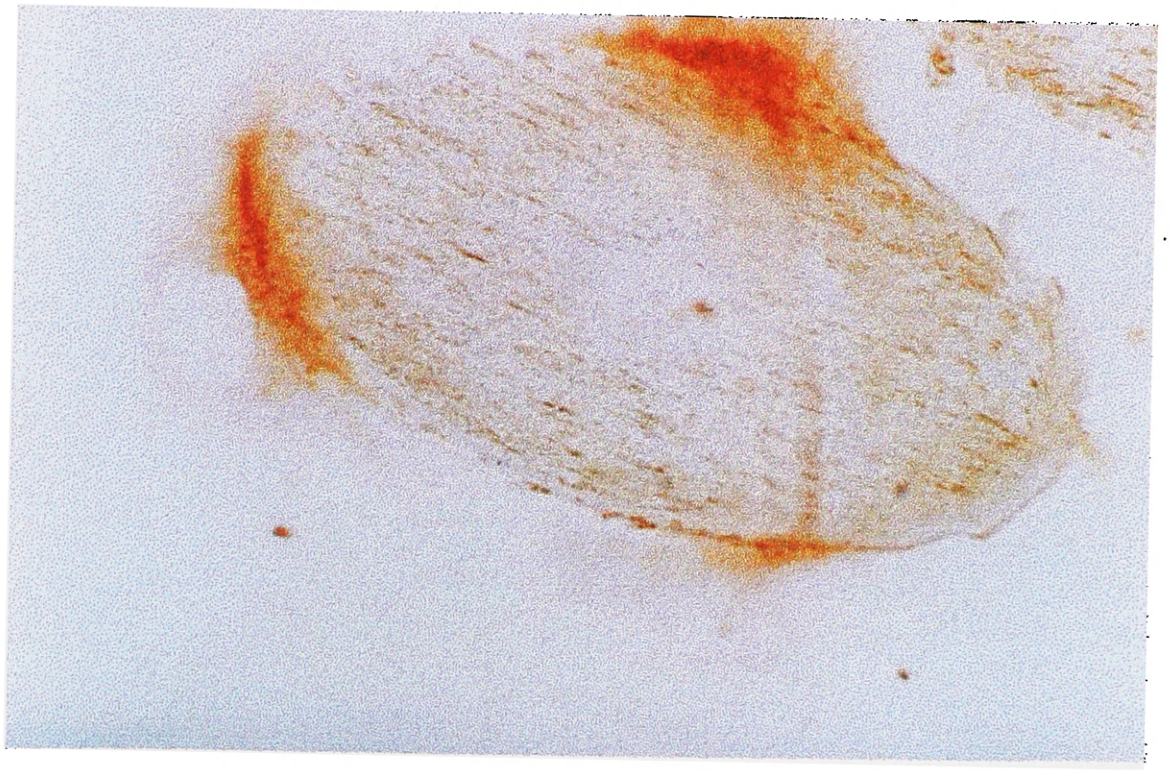


Figure 9: Photographs of (A) Negative hair, (B) Fortified positive hair; hydrocodone, (C) Case 981426, and (D) Case 981743 (Stained with DAB)

C.

D.

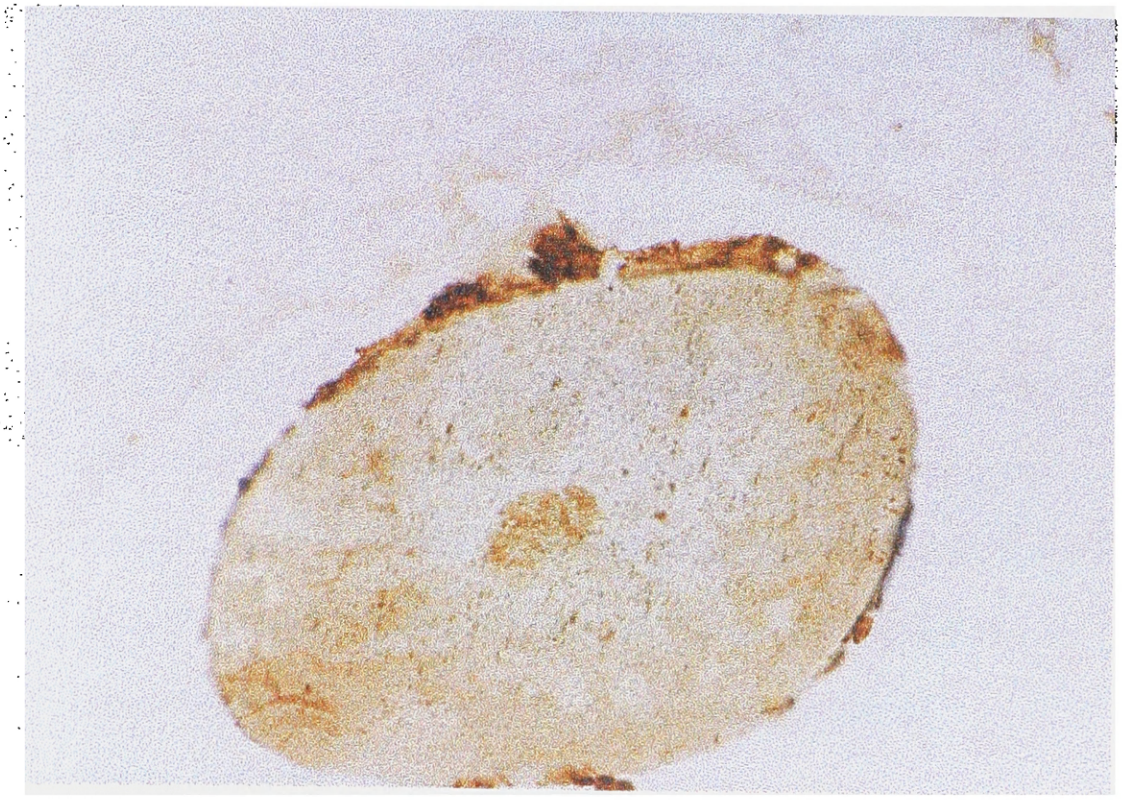


Figure 9: Photograph of (A) Negative hair, (B) Fortified positive hair, hydrocodone, (C) Case 981426, and (D) Case 981426 (Stained with DAB)

Appendix One

Procedure for Drug Analysis in Hair: Decontamination, Digestion, and Extraction

I Decontamination Procedure

A. Hair sample is removed from hair collection kit and approximately 10-15 milligrams of the proximal portion of the hair shaft is placed into a 10 milliliter screw-capped test tube. The weight of the hair sample is recorded.

B. 2 milliliters of de-ionized water is added to the hair sample with constant agitation for a contact period of 15 seconds. The water is decanted off the hair sample and collected in a 10 milliliter screw-capped test tube. This process is repeated once more (for of total of 4 milliliters of wash). Both washes are combined in one test tube. The hair specimen is allowed to air dry overnight prior to being digested. The wash is extracted as described below.

C. 6 milliliters of n-butyl chloride is added to the 4 milliliters of wash in the screw-capped test tube. The tube is placed on a rotary shaker for 10 minutes, centrifuged at 3000 rpm for 5 minutes, and the n-butyl chloride is decanted into a 10 milliliter conical tube. The solvent is evaporated to dryness with the aid of nitrogen in a warm water bath (40° C).

D. 350 micro-liters of HPLC grade methanol is added to the conical tube with

quick vortex (10 seconds). 300 micro-liters of the sample is place in EMIT sample cups and 50 micro-liters of the sample is place in the GC/MS auto-sample vials for further analysis.

II. Digestion of Hair

A. 1 milliliter saturated sodium borate solution is added to the 10-15 milligram hair sample (after decontamination) with quick vortex (10 seconds). 2 milliliters of sodium hydroxide is added to the hair and the tube is placed in a warm water bath (40° C) with constant agitation (using a micro-spin-vane) for 3-4 hours. The digestion time will vary depending on the thickness of hair being analyzed. The tube is removed from the water bath when the hair fibers appear to be translucent and begin to curl. Upon removal from the water bath, the tube is centrifuged at 3000 rpm for 10 minutes, and the supernatant is collected in a 10 milliliter screw-capped test tube.

III. Extraction of Drugs from Hair (Analysis of Basic Drugs: Using a Liquid/Liquid Extraction)

A. 3 milliliters n-butyl chloride and 200 micro-liters internal standard are added to the digestion supernatant and the tube is placed on shaker table for 10-15 minutes and centrifuged for 5 minutes. The supernatant is collected (n-butyl chloride) in a 10 milliliter conical tube. 3 drops (approximately 50 micro-liters) of 1% methanolic hydrochloride is added to the tube and the solvent is evaporated with the aid of nitrogen in a warm water bath (40° C).

B. 350 micro-liters of HPLC grade methanol is added to the conical tube with quick vortex (10 seconds). 300 micro-liters of the sample is place in EMIT sample cups and 50 micro-liters of the sample is place in the GC/MS auto-sample vials for further analysis.

IV. Extraction of Drugs from Hair (Analysis of Acidic/Neutral Drugs: Using a Liquid/Liquid Extraction)

A. Pipette 2 milliliters of the digestion supernatant into a 15 milliliter screw-capped test tube. Add 200 micro-liters internal standard and 1 milliliter of saturated ammonium chloride solution. Quick vortex and add 8 milliliters of ethyl acetate into tube with gently agitation for 1 minute. Centrifuge 10 minutes at 3000 rpm and decant upper solvent to conical tube. Evaporate to dryness with the aid of nitrogen in a warm water bath.

B. Add 0.5 milliliters of hexane to each tube with quick vortex. Add 50 micro-liters acetonitrile with quick vortex and centrifuge for 1 minute at 1000 rpm. Aspirate and discard the upper solvent layer and transfer acetonitrile layer to auto-sampler vials.

V. Extraction of Drugs from Hair (Benzoglecognine Derivitization: Using a Liquid/Liquid Extraction)

A. Add 1 milliliter pH 7 buffer and 6 milliliters 9:1 chloroform: isopropyl to the digestion supernatant. Agitate on shaker table for 5 minutes. Centrifuge (5 minutes at 3000 rpm), decant lower layer to 10 milliliter

conical tube and evaporate to dryness with the aid of nitrogen in a warm water bath.

B. Add 100 micro-liters of DMF and DMF dipropylacetal to the conical tube and reflux 30 seconds with air cool condenser. Cool. Pipette 1 milliliter sulfuric acid (1 normal) with quick vortex, and 3 milliliters n-butyl chloride. Vortex and centrifuge, discard upper layer. Adjust pH to 9 with carbonate buffer (approximately 100 micro-liters). Add 50 micro-liters chloroform to conical tube and vortex. Transfer to auto-sampler vial for further analysis.

VI. Extraction of Drugs from Hair (Solid Phase Extraction)

A. Collect 2 milliliters digest supernatant in test tube and adjust pH to between 4.8 and 5.5 with acetic acid.

B. Condition the extraction column as follows:

1 x 3 milliliters chloroform; aspirate

1 x 3 milliliters de-ionized water; aspirate

1 x 1 milliliter acetic acid (1.0 molar); aspirate

C. Load sample (digest supernatant) at 1-2 milliliters per minute.

D. Wash column as follows:

1 x 3 milliliters phosphate buffer (100 millimolar, pH 6.0); aspirate;

1 x 1 milliliter acetic acid (1.0 molar); aspirate;

Dry column for 5 minutes;

1 x 3 milliliters hexane; aspirate

E. Elute acidic/neutral drugs using 2 x 2 milliliters chloroform (collect eluate at 5 milliliters per minute); evaporate to dryness

F. Extract and analyze acidic/neutral drugs as follows:

Add 1 milliliter hexane and 1 milliliter methanol:water (80:20) to the eluate with vortexing. Centrifuge for 5 minutes at 3000 rpm, aspirate and discard upper layer (hexane). Evaporate to dryness. Reconstitute with 100 micro-liters ethyl acetate.

G. Wash Column with 1 x 2 milliliters methanol; aspirate; dry column for 5 minutes.

H. Elute basic drugs with 1 x 2 milliliters methanol: ammonia (98:4); collect eluate at 1-2 milliliters per minute.

I. Extract and analyze basic drugs as described below:

Add 3 milliliters de-ionized water and 250 micro-liters chloroform to eluate and vortex. Centrifuge, aspirate, and decant as described above. Inject chloroform onto chromatograph.

Appendix Two

Procedure for Detection of Endogenous versus Exogenous Drug Exposure

I. Embedding Protocol

After samples were washed (as described in Appendix One) and dried overnight, they were placed in marked metal screen cassettes (positive, negative, sample), then processed following the procedure below.

A. 3 hour soak in 95% ethanol on rotary table

B. 3 hour soak in 100% ethanol on rotary table (repeat once with fresh reagent for a total of 6 hours)

C. 1.5 hour soak in methyl salicylate

D. Soak overnight in fresh methyl salicylate

E. 3 paraffin soaks (each for 3 hours) changing to fresh reagent each time

After embedding, the hair was removed from the cassettes and placed in plastic paraffin cups then covered in paraffin. The paraffin blocks were sectioned in 15 nanometer slices and mounted onto microscope slides. The mounted slices of hair were carried through the monoclonal protocol described below.

II. Monoclonal Antibody Protocol

A. Deparaffinize slides as follows:

10 minutes in xylene (x3)

3 minutes in 100% ethanol (x3)

3 minutes in 95% ethanol (x3)

3 minutes in 70% ethanol (x3)

B. After deparaffinization, rinse slides once in 0.1 molar PBS, repeat with fresh reagent and soak for 5 minutes.

C. 5 minute soak in PBS/BSA (250 milliliters 0.1 molar PBS + 1.25 grams BSA)

D. 20 minute soak with 4% NHS (12 milliliters TAB + 5 milliliters normal horse serum; TAB = 50 milliliters tris(0.1 molar)/BSA + 500 micro-liters 10% sodium azide in distilled water)

E. Blocking serum was removed and primary antibody was applied (morphine monoclonal antibody with a Kd of 2 nanomolar for codeine; 120 micro-liters antibody (at 500 micrograms/ 0.53 milliliters) in 2.88 milliliters PBS). Slides were incubated overnight at 4° C.

F. Rinse in PBS, refresh reagent and repeat for a 5 minute soak.

G. 5 minute soak in PBS/BSA

H. Incubate for 1 hour with secondary antibody, horse-anti-mouse (4 milliliters TAB + 10 micro-liters antibody (1:400 dilution))

I. 5 minute soak in PBS, followed by 5 minute soak in PBS/BSA

J. Incubate for one hour in ABC reagent (5 milliliters PBS/BSA + 1 drop vectastain elite A + 1 drop vectastain elite B - cover and let sit 30 minutes; dilute 1 milliliter of this solution with 5.7 milliliters PBS/BSA = ABC reagent)

K. Rinse with PBS, then soak for 10 minutes in fresh PBS

L. Apply DAB and allow to sit for 10 minutes (4 milliliters PBS + 50 micor-liters 0.1 molar imadizole + 1 milliliter DAB + 5 micro-liters hydrogen peroxide)

M. Rinse in de-ionized water. Repeat x2 using fresh water each time.

N. Dehydrate slides as follows:

5 minutes in 70% ethanol

5 minutes in 95% ethanol

5 minutes in 100% ethanol (x3)

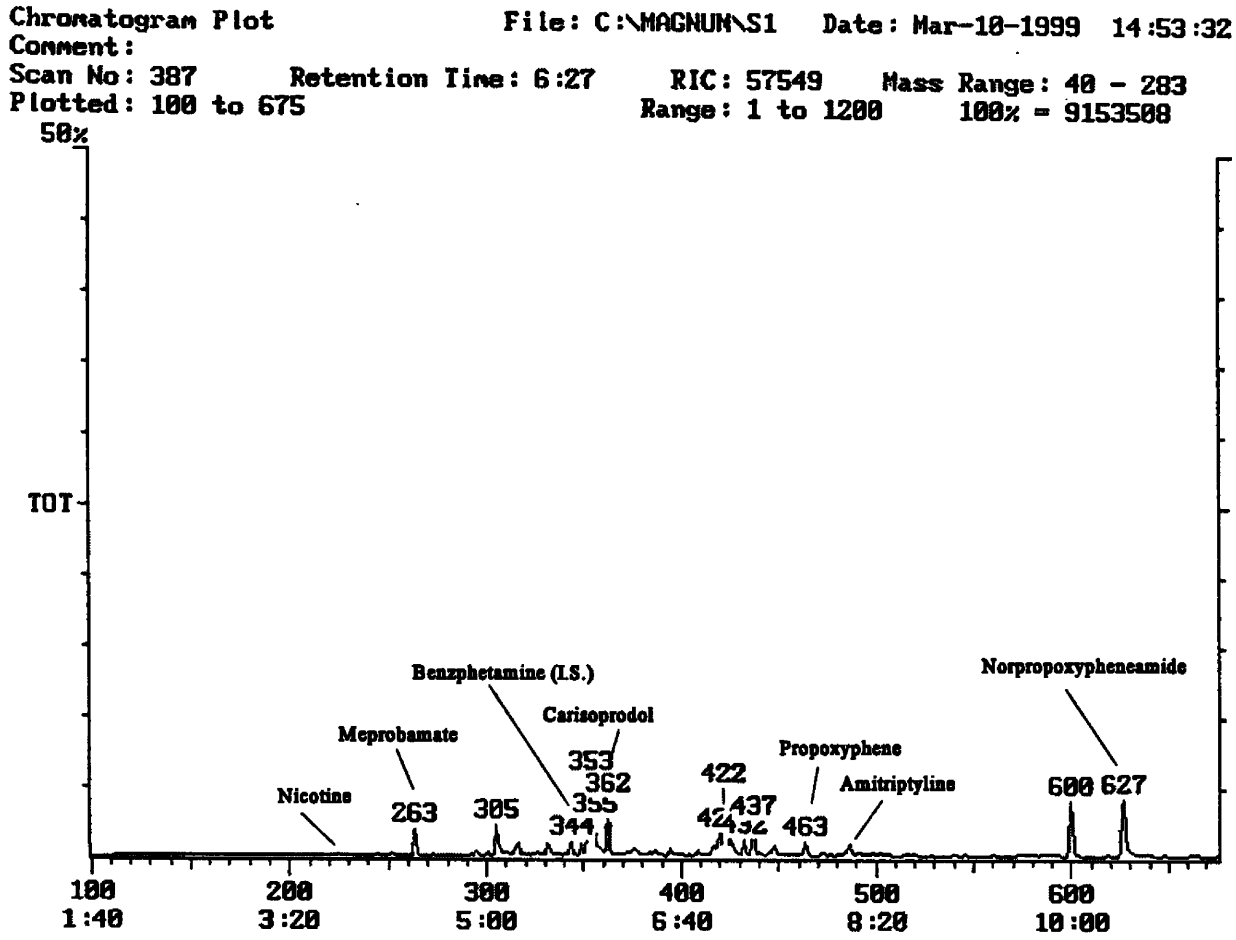
5 minutes in xylene (x3)

O. Coverslip slides

Appendix Three

Chromatograms of Reproducibility Experiment

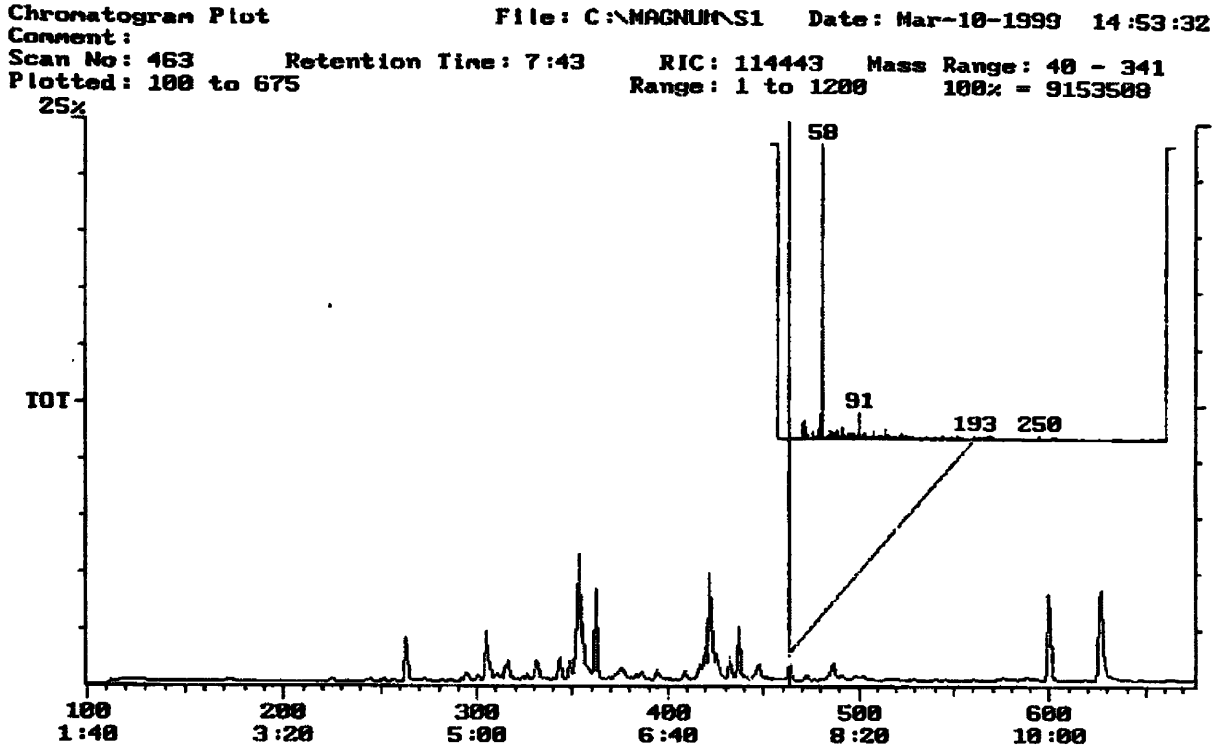
A. Chromatogram of S1

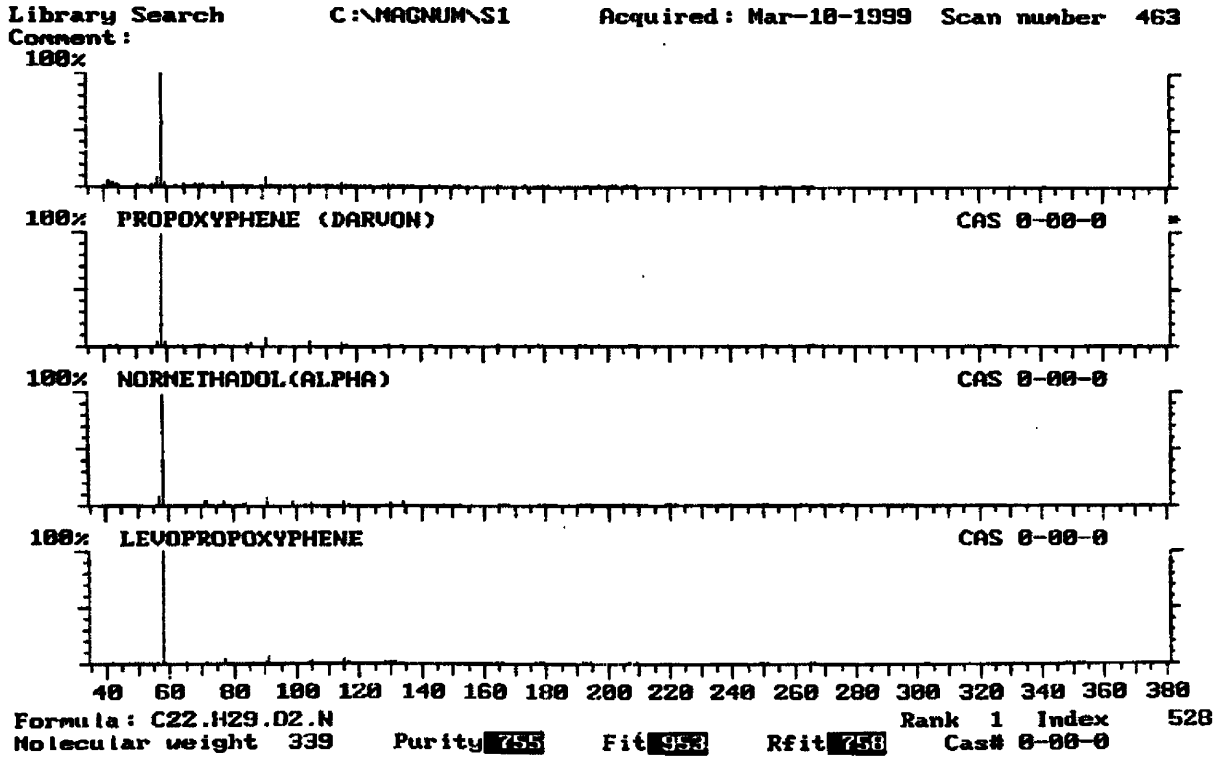


The above chromatogram (A) is the first of two samples of hair from case 990547 which were analyzed to test the reproducibility of the methodology. Sample S1 (chromatogram A) and sample S2 (chromatogram C) were obtained by splitting a 20 milligram sample of the decedent's hair into two 10 milligram portions. Digestion and extraction procedures were run (in parallel) on both specimens. Both chromatograms show identical spectra, with the identification of six drugs and the Internal Standard benzphetamine (designated I.S.). Chromatograms B and D are included to demonstrate two of the three criteria utilized for peak detection. In chromatograms B and C, the fragmentation pattern and retention time of a representative drug (propoxyphene) are shown for S1 and S2. In addition, both samples record a library match with a "Fit" that exceeds the recommended 850 count. Data showing the third criteria,mean signal-to-noise ratio, is not shown.

To further insure instrumental integrity, the reproducibility of the analytical

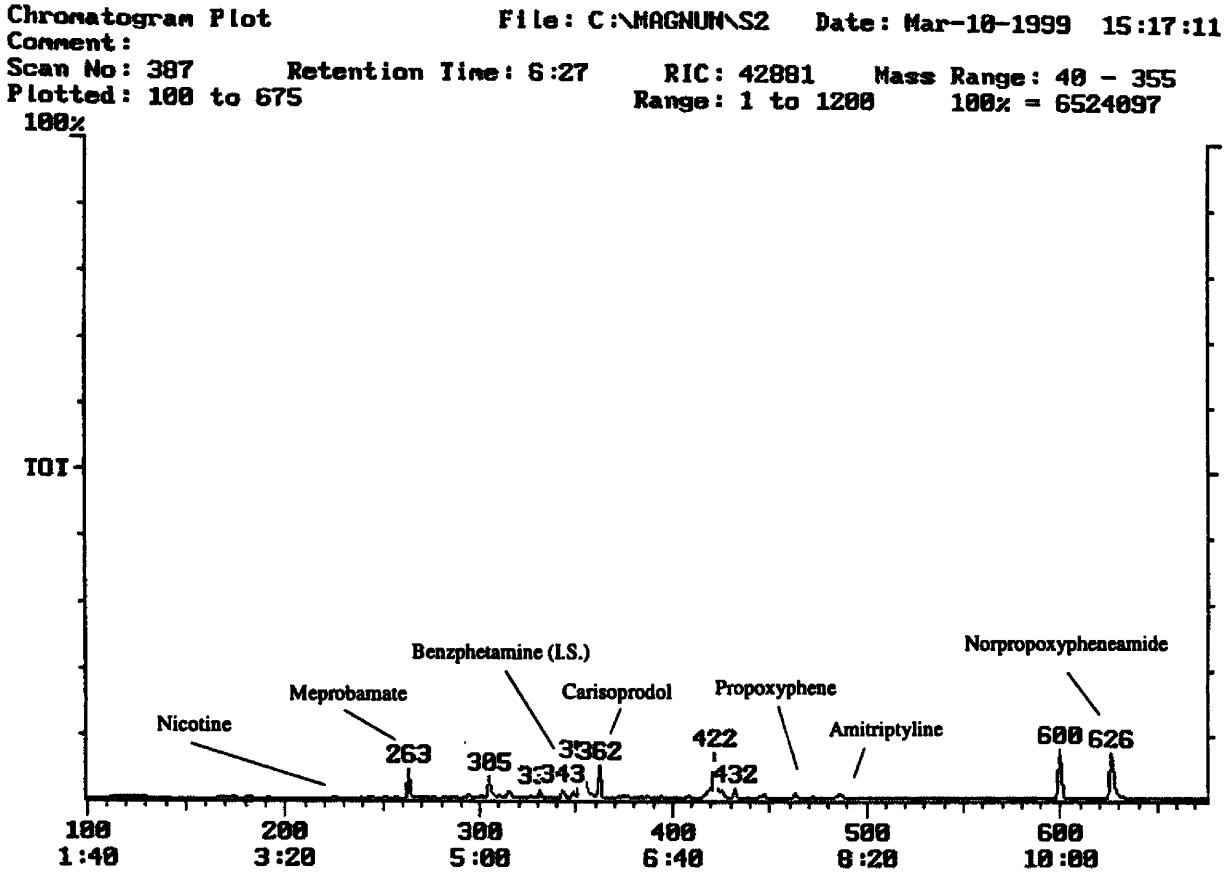
B. Fragmentation Pattern and Retention Time For Propoxyphene in S1





(Notice the "Fit" for the library search exceeds the recommended count of 850 by registering 953.)

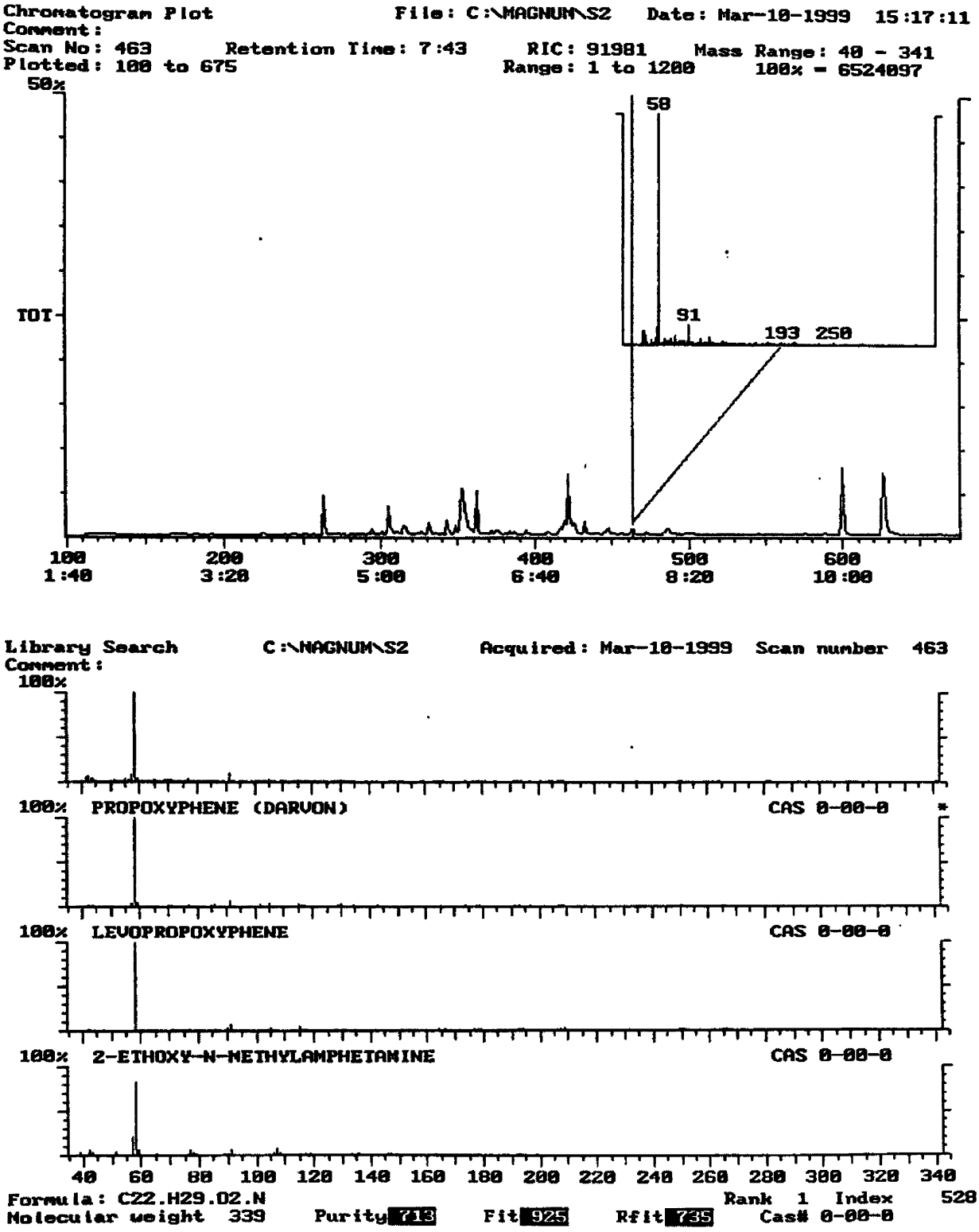
C. Chromatogram of S2



procedure was tested. Upon conclusion of the split sample analysis (as characterized in chromatograms A and C) a second experiment was performed using the same extract (S1) several hours after the original analysis. The extract was stored at ambient temperature upon conclusion of the original analysis until the second analysis was performed. Chromatogram E relates the results of this experiment. All six drugs identified in the original analysis (and the internal standard) were confirmed in the second analysis.

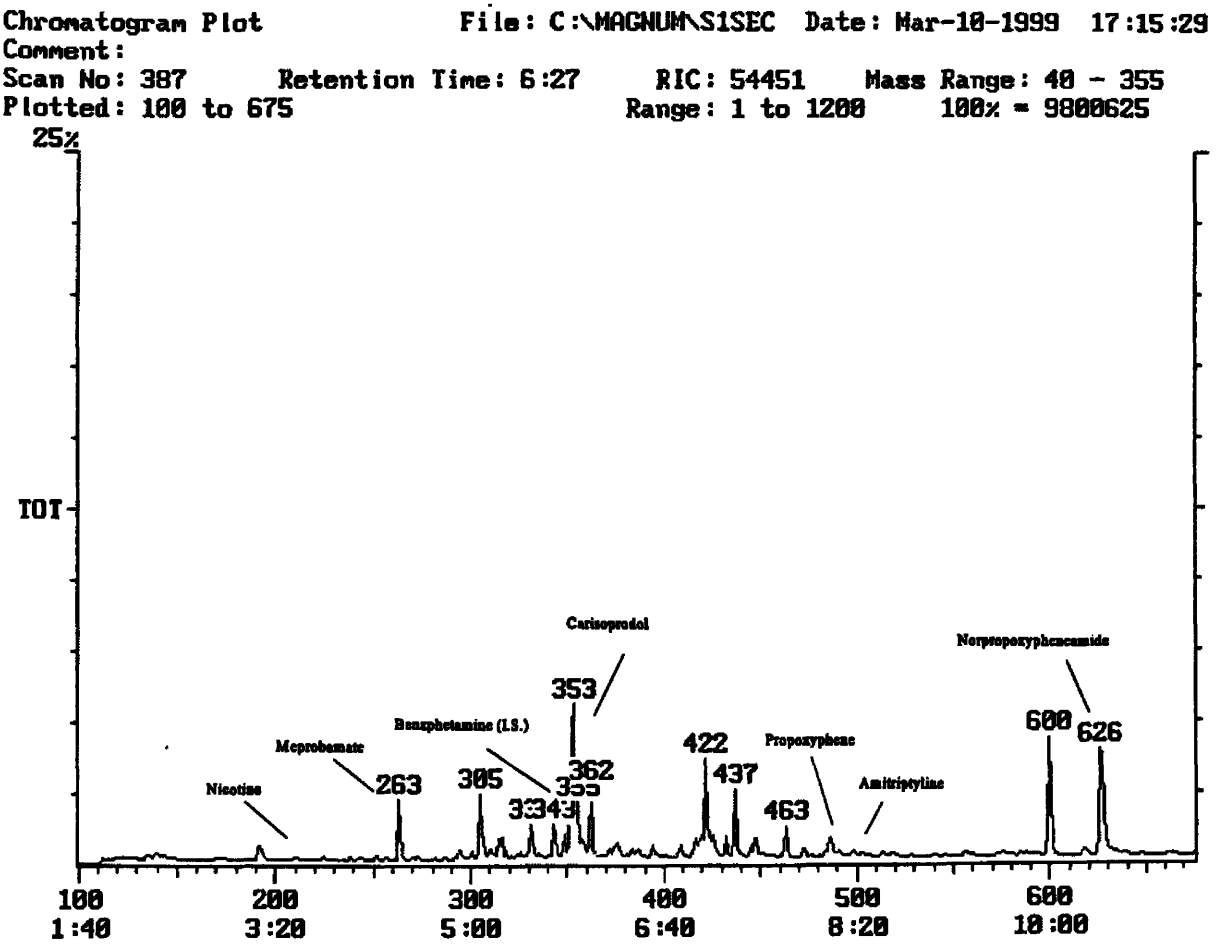
These findings support our belief in the reproducibility of our methodology and analytical procedures.

D. Fragmentation Pattern and Retention Time For Propoxyphene In S2



(Notice the "Fit" exceeds the recommended 850 count by registering 925.)

E. Second Analysis of Fraction S1



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